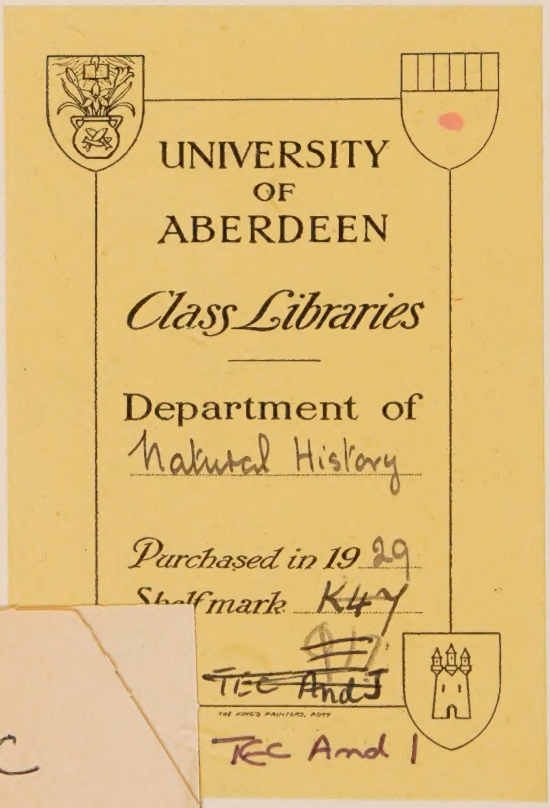

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HOW TO STAIN THE NERVOUS SYSTEM

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A Laboratory Handbook for Students and Technicians

BY

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INTRODUCTION

Technical difficulties in anatomo-pathological research have hindered or checked much work on the nervous system. In many laboratories little attempt is made to investigate unusual diseases of the brain or cord, for the reason, mainly, that the methods necessary for this investigation are unfamiliar and appear to necessitate apparatus and stains other than those which are to hand. But these difficulties are more apparent than real. It is true that certain staining methods are best carried out after celloidin embedding, but this is rarely an absolute necessity, and most of the routine procedures can be performed almost as well on frozen or paraffin sections. There is also a field here for gelatin embedding, but, so far, few attempts have been made to systematise this into a routine method. As it has not been used in the laboratory of the National Hospital, no mention is made of it in this book.

Another difficulty arises from the multiplicity of modifications of some of the best known routine methods. There appear to be about fifty modifications of Weigert's method for myelin sheaths, and although the later Weigert Pal formula is that

most commonly in use, even in it there are minor variations in the times of the various procedures. The well-known methods of Nissl, Marchi and Bielschowsky have also undergone numerous modifications, and the descriptions of these in most text-books do not give sufficient details to enable a beginner to attain success. Still more unsatisfactory, in English text-books at any rate, are the descriptions of neuroglia staining methods. In fact, many of those given are so complicated and so uncertain in their results that a beginner may well despair of success with them.

For these reasons it has seemed worth while to collect in a small space the routine methods in use in the laboratory of the National Hospital, Queen Square, and a few of the other methods which, although of more service in special researches, are of no very great technical difficulty. In this little book an attempt has been made to give sufficient details to enable any worker with the most elementary knowledge of general histological work to obtain satisfactory or even good results at the first attempt. Small details are of even more importance in the histology of the nervous system than when dealing with other tissues of the body, and it is important that a worker should follow the method exactly as described, at any rate until he is thoroughly conversant with it. It will be found that almost all the methods have been modified to some extent from the classical descriptions. The most im-

portant of these modifications is the standardisation of temperatures, and the use of fairly high temperatures such as 50° C. for certain stains. This not only shortens the times of staining, which are often unduly long, but in our experience often gives the stain a more selective action. Another modification, which originated with Mr. Anderson, is the extensive use of calcium hypochlorite as an oxidising agent, both in the ripening of solutions of hæmatoxylin and carmine, and in mordanting with chrome salts. A certain number of these modifications have already been published by him. Others are given here for the first time.

It remains to be said that all the methods detailed here have been tried out thoroughly in the laboratory of the National Hospital and have been found to give almost constantly good results. Many have been in use for years without modification. Others, as for example, the Marchi methods are still undergoing a process of evolution. Perfection is difficult to attain, and new or improved methods are still needed to reveal many histological structures. But if a beginning is made with those given here, the laboratory worker will, we hope, be saved both time and disappointment.

It is hoped that this little book will be found useful, not only by the laboratory technician, for whom it is primarily intended, but also by those learning neuropathology and even by research workers. It is meant to supplement, rather than

to replace, more exhaustive works such as *The Microtomists' Vade-mecum* to which, and to the excellent laboratory manuals of Spielmeyer and of Roussy and Lhermitte it owes much.

The method adopted in it is that of Roussy and Lhermitte's manual. Each method is given in full detail and this is followed by a résumé for later reference. Formulæ of some of the most commonly used fixatives, mordants, and stains are given in the Appendix. So far as possible the methods detailed are adapted for tissue which has been originally fixed in formol-saline, seeing that this remains the best fluid for fixing the nervous system in bulk, and for anatomo-pathological research this is an essential preliminary.

J. G. GREENFIELD.

HOW TO STAIN THE NERVOUS SYSTEM

CHAPTER I

FIXING AND CUTTING THE BRAIN AND SPINAL CORD

At the autopsy, the brain and cord should be removed together, or at any rate exposed throughout their entirety. This can only be done if the brain is removed from behind and the occipital bone removed down to the foramen magnum. It is usually best to cut the cord through at the level of the third or fourth cervical segment and to remove the upper part with the brain, and then to remove the cord in its dural covering. When removing the cord, care should be taken to avoid bending it or kinking it. It is then laid out flat, and the dura mater is cut up the middle of both front and back, and several cuts made across each half of it. This is done to avoid kinking of the cord by contraction of the dura mater during fixation, and to ensure rapid penetration of the cord by the fixative. It is not usually necessary to suspend the cord. It may be laid slightly curled on the bottom of a large container. The brain should be suspended

in the container by a string which is passed under the basilar artery. Before this is done it is advisable to make incisions into the ventricles through the infundibular region and through the corpus callosum, so that the fixative may penetrate easily into the ventricles. It is very inadvisable to cut the brain up at the post-mortem as the shape of the different parts is altered and valuable material may be rendered useless in this way.

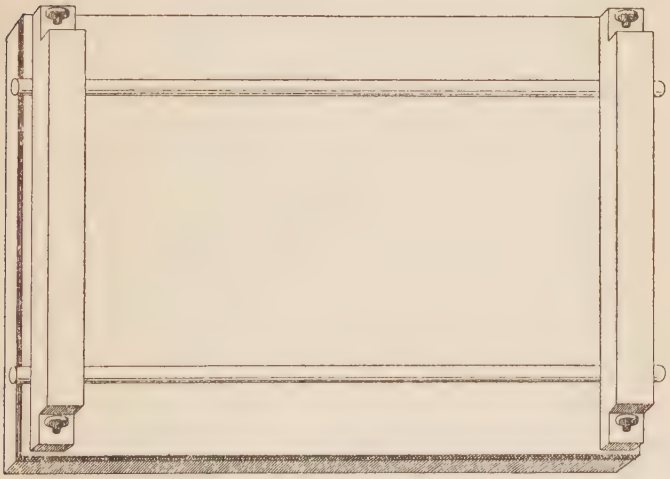
The fixative generally used is 10 per cent. formol in 1 per cent. sodium chloride solution, of which 4 or 5 litres are necessary for the fixation of the whole brain. This should be changed after twenty-four hours. At the end of the first week the brain should be sliced so that the fixative may penetrate more thoroughly ; a few days more in the formol saline complete the fixation ; the tissue is then ready for further investigation.

Slicing the Brain

The correct slicing of a brain is of too great importance for haphazard methods. In order to get even slices, and especially for investigation of the basal ganglia, a simple and ingenious machine has been devised, which is made in the following way. The material for its construction is easily procured.

The base should be a flat piece of hard wood, $10\frac{1}{2}$ inches long, $7\frac{1}{2}$ inches wide, and at least 1

inch thick. In addition we need a sheet of aluminium of the same size, and about $\frac{1}{8}$ inch in thickness, two cross pieces from the same kind of wood $7\frac{1}{2}$ inches long, $\frac{3}{4}$ inch wide and $\frac{1}{2}$ inch thick, four brass thumbscrews about $\frac{3}{16}$ inch



thick, having $1\frac{1}{2}$ inches of screw thread, four small brass washers with holes large enough to slip on the screws, and some pieces of glass rod, ranging from 3 to 10 mm. in diameter, according to the thickness of slices required.

The aluminium sheet is first screwed on to the flat piece of wood, then a hole is tapped at each corner with a thread to take the thumbscrews.

Holes are then bored in the ends of the cross pieces large enough to allow the screw to pass through easily. The washers are slipped on the thumbscrews, and the screws are passed through the cross pieces and screwed into the base. The glass rods are held firmly by their ends between the base and the cross pieces. They may be set at any distance from each other and are kept in place by tightening the thumbscrews.

The diagram shows the machine completed.

For most purposes the brain as well as the brain stem and cord should be cut horizontally. In order to have some definite level for the first slice through the brain, it is usual to select the plane which passes immediately below the splenium and genu of the corpus callosum. The flat surface of brain thus obtained is laid on the base of the slicing machine, between the glass rods, pressed firmly but carefully down and the knife run through it, sliding along the glass rods all the way. When the top piece is lifted away there will then be an even slice lying between the rods on the slab. In this way a series of even slices of the same thickness can be cut.

In slicing a brain to find out the situation of a tumour or other disease, slabs 10 mm. thick are convenient. The most usual thickness for pieces which are to be examined histologically is from 3 to 5 mm. The best knife to use is a long slender ham knife with a parallel edge and back. These can be bought in various lengths and widths.

The most convenient is one with a 10-inch blade and 1 inch wide.

When the brain is cut for any special purpose, the slices are kept in proper order and numbered with indian ink. Photographs of the slices may be taken at this stage if desired. Portions of tissue needed for further investigations may be cut and marked with indian ink according to the slice from which it was taken. Attention should be drawn to the fact that it is necessary to mark right or left side of the brain as well as the number of the slice. A few seconds are necessary to allow the ink to dry, after which it is indelible. The pieces are then put into the various solutions, according to the method of investigation intended. The remaining pieces may be preserved in formol saline indefinitely, or in 70 per cent. alcohol if it is desired to stain the Nissl granules of the nerve cells.

Cutting up the Cord

It is sometimes necessary to cut sections from every segment of the cord. In this case it is necessary to know the exact position of every piece. For this purpose, the cord should be first cut through horizontally between each segment, that is, between the origins of each of the pairs of nerve roots. It is best to begin, not at the top of the cord, but at the junction of the cervical with the dorsal segments, as this level is easily localised,

whereas it is not easy to say exactly where the cervical cord joins the medulla. The best indications as to the position of the first dorsal segment are: (1) That it is the place where the cord becomes rounded again after having widened out in the lower cervical region. (2) The anterior root of the first dorsal segment is definitely smaller than that of the eighth cervical segment, whereas the posterior root is almost as large. Thus the anterior root of this segment is considerably smaller than the posterior root. (3) When the cord is cut across between the eighth cervical and the first dorsal roots, the anterior horn of grey matter runs out laterally towards the side of the cord, whereas at the lower end of this segment, as in all the other dorsal segments, it has no such lateral projection. Another point to remember is that the lowest four cervical segments have large anterior roots, coming off the cord as a broad ribbon. The upper four cervical segments have much thinner roots. In many cases it is only necessary to take pieces from the cervical and lumbar enlargements, and the mid-dorsal region, but it is always best to know from which segment they are taken.

Preparing Tissue for Section-Cutting

There are three methods in general practice by which sections may be cut for staining purposes. *Firstly*, the tissue may be frozen and cut in the

freezing microtome; *secondly*, it may be infiltrated with paraffin; and *lastly*, it may be infiltrated with celloidin. In most pathological laboratories paraffin is invariably used, but for the central nervous system this is not so satisfactory as celloidin, partly owing to the shrinkage which occurs with paraffin. Frozen sections are the most natural, but they have the disadvantage of losing the meninges and the outer blood-vessels; but this is the quickest, and for a number of methods practically the only way. These two methods will therefore be chiefly dealt with.

Threading the Cord

Before proceeding further, it will be best to explain the method of keeping the segments of the cord in order, as this is essential if sections are to be stained in sequence.

Firstly, the cord is cut through at the level of each segment.

Secondly, portions are taken from every segment for whatever staining methods are to be used. As each portion is cut from the segment it is laid on the bench in order. Portions may be taken (1) for celloidin or paraffin embedding without mordanting, (2) for the Weigert Pal method, or (3) for the Marchi-Busch method, or for other methods which require preliminary mordanting.

Thirdly, when the portions are all cut, they are threaded on a piece of fine silk-worm gut or

horse-hair. The worker may start at either end of the row of pieces, but he must see that each one is put on in proper order. When threading the cord it is best to tie a loop at one end of the thread, the first portion is pulled close up to the loop, and the other pieces are threaded on and run up the thread till they almost touch each other. When they are all on, the lower end is passed through the loop and tied.

Points to Remember

1. Each portion must be threaded the same way.
2. Do not pass the thread through the region of the horns, except for the Weigert Pal method.
3. Thread the spare portions in order for frozen sections, or for other methods.

CHAPTER II

CELLOIDIN SECTIONS AND STAINING METHODS

Good celloidin is essential. Pyroxylin may be used, but is not so good as Schering's celloidin which is sold in one-ounce bottles. It is taken from the bottle, washed well in distilled water, dried at a low temperature, preferably in the incubator at 37° C., and then dissolved in absolute alcohol and ether. Some workers use equal parts of alcohol and ether, but the author has found that six parts of ether to four parts of alcohol makes the best solvent.

Celloidin should be made up in three different strengths—thin celloidin 2 per cent., medium 4 per cent. and thick 8 per cent. The bottles used for stocking celloidin solution should be wide mouthed with glass stoppers and of a capacity to suit the amount of work done in the laboratory. A spare bottle should be kept for dirty celloidin to be stored in, as it may be cleaned and made fit for further use by a simple process to be described later. When dissolving celloidin it should be stirred as often as possible until an even solution is obtained; otherwise if left for several hours

unstirred the celloidin sets in a solid mass at the bottom of the bottle.

Celloidin Infiltration

Celloidin infiltration is most often used for tissue to be stained by the following stains: (1) Alum or iron hæmatoxylin and a counter-stain, or toluidin blue for Nissl bodies. This work is usually termed "ordinary celloidin." (2) Myelin staining which is donè with an acid hæmatoxylin after mordanting in bichromate or chrome salts. (3) Osmic acid staining for early degeneration. For the two latter methods, special mordanting before infiltration is needed.

Let us consider "ordinary celloidin" first. Pieces of tissue are taken after fixation and placed for twenty-four hours in equal parts of spirit and distilled water, then twenty-four hours in spirit, twenty-four hours in absolute alcohol, and not less than twenty-four hours in equal parts of absolute alcohol and ether. If the pieces are large and numerous, they should remain in alcohol and ether forty-eight hours and the solution changed once; this ensures the tissue being thoroughly soaked. The pieces are now ready to be placed in 2 per cent. celloidin and should be left in this for seven to ten days according to size and thickness. The 2 per cent. celloidin is then poured off and replaced with 4 per cent. celloidin; the pieces stay in this for four to seven days,

after which they are embedded in 8 per cent. celloidin.

Celloidin-Embedding

Embedding may be done either in a flat glass dish or in an enamelled developing dish. Whichever kind is used it should be large enough to hold the pieces of tissue for embedding easily, so as to allow them to be kept apart; this obviates the risk of the tissue being cut when being removed from the dish. The 8 per cent. celloidin is first poured into the dish to a depth which more than covers the thickest piece of tissue. The pieces are then set out and pressed gently down to the bottom of the dish. If any special order is desired they should be placed in the dish in that order.

N.B.—By "spirit" is meant colourless commercial methylated spirit.

When all the pieces are embedded, the dish is covered over completely with a glass plate and should be left so for twenty-four hours; this will allow all the bubbles to rise from beneath the tissue. The glass plate is then moved slightly to one side to allow the alcohol and ether to evaporate slowly and the celloidin to set. Quicker setting may take place on that side which is uncovered; if this occurs the glass plate should be moved so that the other end of the dish may be exposed. Once the whole surface has set, the plate may be removed alto-

gether, the celloidin will then begin to harden very quickly. It is left exposed like this until the celloidin feels like hardened leather.

The pieces can now be separated and taken from the dish. To do this cut the celloidin between each piece of tissue with a sharp knife and also run the knife all round the edge of the dish. If the worker follows the method just given each piece is taken out separately. If he desires to take the pieces of embedded tissue out in one slab the knife is run round the edge only. Whichever method is chosen it is done in the following way. Cut a piece of spare celloidin out so that the finger may be inserted and used as a lever to free the tissue from the bottom of the dish; once a start has been made it is easy to remove the remainder. Great care is necessary in doing this or the tissue may be broken. Each separate bit of tissue is mounted on a wooden block preparatory to cutting into sections.

A most important precaution must be mentioned here. When the tissue to be embedded has been mordanted with chromates or osmic acid it is necessary to place a piece of tin foil on the bottom of the dish before the 8 per cent. celloidin is poured in. This is most essential, because the mordanting makes the tissue very brittle, and if placed directly on the bottom, it may adhere firmly to it and break when one tries to remove it.

Mounting on Blocks

Each piece of tissue is trimmed before it is mounted by cutting away the spare celloidin, leaving a small margin at the edge of the tissue. No matter what the shape of the tissue may be, the celloidin should be cut so that the edges are straight, but with as little spare celloidin as possible.

A word of advice as to the nature and treatment of the wood is necessary here. Wood used for the mounting of tissue must be of the hardest kind, such as elm or oak, preferably the former. The wood must be soaked in spirit for a few days before use in order to get rid of the resin and tannin. It is allowed to dry and then cut into blocks large enough to take the pieces of embedded tissue easily, so that the tissue has a firm bed to rest on. The block must have two straight parallel edges, so that the clamp of the microtome for holding the blocks may get a firm grip. When the block has been cut, one side should be rubbed smooth with glass paper to allow the name and number of the block to be written on it with indian ink. As the blocks are named and numbered the pieces of tissue corresponding are stuck on with 4 per cent. celloidin. This is done either by dipping the tissue in the celloidin or by pouring some on the block; whichever method is used the tissue must be pressed firmly down on the block so that no air bubbles remain

beneath. They are then left on the bench to dry and harden. Care should be taken to see that the edges of the celloidin in which the tissue is embedded are lying down flat and firmly fixed. When the celloidin joint is quite hard the blocks are stored in equal parts of spirit and distilled water until the worker is ready to cut them.

One important point about the wooden blocks must be mentioned. Very often pieces of tissue will be so large that when they are mounted it will be impossible to get the blocks into the clamp of the microtome should they be simply mounted on flat pieces of wood. This difficulty may be easily overcome: some workers have screwed a smaller piece of wood underneath; but there are great disadvantages in this method, for if the screws are iron they will rust and fall out, and even brass screws will become loose after a time. The best method is as follows:—

The wood used for large pieces should be no less than 1 inch thick. One half of the thickness is sawn away, excepting an oblong piece at one side small enough to go into the microtome clamp. The side at which this piece must be left is that which will encounter the knife first when sections are cut. As to the size and position of the projecting portion the worker must use his own discretion, according to the size of tissue to be mounted and the make of microtome used.

CELLOIDIN SECTIONS

Microtome and Knives

By far the best form of microtome for celloidin work is the sledge type in which the knife is held on a heavy block which slides in a groove. The best microtomes of this kind are made by Reichert of Vienna and Jung of Heidelberg. Celloidin sections can be cut on microtomes of the Minot type if provided with a knife-holder, which holds the knife in a slanting position. The Cambridge Universal flat cutting microtome can also be used, but none of these models is as satisfactory as the sledge type. Good sections cannot be cut except with a good and really sharp knife. The best to use for cutting celloidin sections is one which is moderately hollow ground on one side only. The most generally useful length of knife is 20 cm., but for small blocks of tissue 16 cm. may be sufficient.

Sharpening the Knife

Section-cutting takes the edge off the knife very quickly, and as it is not always convenient to send it to be sharpened every time the edge becomes dull, a few words of advice are given regarding the sharpening and stropping. It is necessary to have a good large hone, 8 to 10 inches long and $2\frac{1}{2}$ inches wide, with a smooth grinding surface. A yellow Belgian hone has been found to be the best, using soap lather instead of oil. It

is best to have two strops, an ordinary flexible razor strop and a solid one, both of which should be at least $2\frac{1}{2}$ inches wide. For knives which are hollow ground on one side only a special honing back is needed, which is slipped on to the knife before it is honed.

The surface of the hone and the soap lather must be free from grit. When a good lather has been produced on the hone, take the handle of the knife in the left hand and place the knife gently on the hone with the concave side down. Holding the end of the knife with the right hand draw it diagonally across the hone from heel to toe, that is, from right to left, keeping it pressed lightly on the hone. Slide it very lightly back and repeat this process forty to fifty times. The honing back is then taken off, and the knife is laid with the flat side to the hone and is drawn in the opposite direction several times. The latter process takes off most of the wire edge caused by the former movement.

Stropping the Knife

The knife is now ready for stropping; this should be done on the solid strop. One end of the strop is rested on the bench and the knife is drawn across from heel to toe, turned over on its back and drawn across in the opposite direction. In this case the direction of movement is always towards the back of the knife.

This is repeated until all the wire edge is removed. To test the sharpness of the knife take a hair and try to cut it in two about half an inch from where it is held. If this can be done the knife is sharp and will cut good sections. The flexible razor strop may be used for occasional stropping during the period of cutting a batch of tissue blocks. The worker must bear in mind that a microtome knife has a very delicate edge, therefore at no time must it be forced down on the hone or strop. If the advice given is carefully followed the knife may be used a long time before it has to be sent for grinding and resetting.

Sometimes a piece of gritty substance may be embedded in the tissue, and before the worker can prevent it the knife will have several notches on it. This necessitates regrinding at once.

Cutting Celloidin Sections

Section-cutting may start any time after the tissue has become hard and fast on the blocks. It is better to leave them in the spirit and water mixture for at least half an hour, as the harder the tissue the better it will cut. Celloidin sections can be and have been cut at 4 microns, but 6, 8 and 10 microns are about the average in ordinary celloidin work. One must remember that a perfect section at 8 microns is in every way better than a ragged one at 4. In cutting

sections the worker will have to adapt himself to the kind of microtome at his disposal, but in all cases the knife must be on the slant and is drawn through the tissue from heel to toe ; the greater the slant of the knife the better the cut will be, therefore for large pieces of tissue it is necessary to have a long knife.

There are two methods by which celloidin sections may be cut, but in both the knife and the tissue must be kept wet with spirit, therefore a bottle of spirit should be kept at hand or a continuous drop of spirit on to the knife arranged.

Method 1.—Sections are cut by drawing the knife through the tissue in one continuous movement, the spirit being poured on the whole time. This is a useful way, especially when big pieces of tissue are being cut, because, as the knife is drawn through, the spirit washes the section down in a roll. The section is then dropped into a dish of spirit where it will usually unrol itself ; if not, a few dabs with a small soft brush will straighten it out, when one can see if the section is good enough to keep for staining. With a little practice the worker will know as the section is being cut whether it is going to be good or not. It is impossible to give hard and fast rules for cutting celloidin sections as sometimes they will not cut in the manner explained ; if this be so the worker should try the following method.

Method 2.—The cutting of the section is done

by a forward and backward movement of the knife, the former always exceeding the latter. The spirit is not used so freely in this method, only enough to keep the knife and tissue wet, and good sections may be obtained at every cut; moreover, if the spirit is dropped on in the right amount the section slides up the knife, thus allowing the worker to see exactly what it is like. Whichever way the sections are cut they are put into spirit immediately. If the sections are in no particular order one dish of spirit will do to hold them as they are cut from the different blocks. If they are to be stained in series or sequence they are put into small bottles and numbered, or they may be put into small dishes made for that purpose. These are square blocks of glass hollowed out on one side, and are very useful because they can be placed one upon the other. When the sections are all cut they may be stained immediately or stored in spirit indefinitely.

For handling sections the following implement may be used. This is a glass hook made from a piece of glass rod drawn to about the thickness of a steel knitting needle over a fish-tail burner; it is cut in the centre with a file and the rough edges smoothed in the flame. About 1 inch of the thin end is then bent at an obtuse angle. The smoothing of the end must be done without any thickening or it will damage the sections.

Staining Celloidin Sections

Ordinary celloidin sections may be stained in various ways :—Hæmatoxylin, with a counter-stain, for general purposes ; toluidin blue for Nissl bodies ; and neuroglia stains.

Hæmatoxylin Staining

1. *Alum hæmatoxylin.*—Various formulæ have been made by different histologists, but the two in most common use are Ehrlich's and Delafield's. At the National Hospital, Queen Square, the hæmatoxylin in general use is made according to the author's formula and gives good and constant results with the advantage of being ready for use as soon as it is prepared.

2. *Iron hæmatoxylin.* — Weigert's formula or the iron hæmatoxylin formula given in the Appendix may be used. We have found the latter preferable. The sections are transferred from spirit to water for about a minute and then they are transferred to the stain, where they remain for a time according to the hæmatoxylin used, usually about five minutes. When they have been in the stain the allotted time, they are transferred to tap water which is immediately changed. This change of water is essential to allow the sections to become blue, so that the nuclei may stand out clear and sharp. The blueing depends on the alkalinity of the water. Some tap water

is definitely alkaline, but in some towns it may be necessary to use distilled water to which has been added a few drops of saturated solution of lithium carbonate. Ammonia may be used, but great care must be taken on account of its tendency to take out the stain. It is much better to overstain with hæmatoxylin and then differentiate with acid alcohol, which is 70 per cent. alcohol and 1 to 2 per cent. hydrochloric acid. This is done by immersing a stained section in acid alcohol for a few seconds and transferring to a fresh dish of tap water. The advantage derived from this is that the nuclei tend to be much darker and sharper, which is essential when counter-staining with Van Gieson.

With celloidin sections Van Gieson is more generally used as, a counter-stain as this stains the connective tissue differentially from the nervous tissue and when properly executed gives a very pretty picture. Iron hæmatoxylin should always be used before this counter-stain. Counter-staining with Van Gieson is not easy and requires a special technique if constant good results are desired. Ernst recommended the proportion of 10 parts solution A to 1 part solution B, the time of use being ten seconds. We use it in the following proportions :—12 parts A to 1 part B for the brain and cord, and 20 parts A to 1 part B for muscles and nerves. With a freshly made stain five to six seconds is long enough to stain.

If a large number of sections are being counter-stained it must be taken into consideration that the stain is getting weaker, owing to the water which is carried into it with the sections; the worker must therefore use his own discretion as to the length of time required for staining. After the requisite time in the stain the sections are transferred to distilled water and moved about in it for a second or two; then into spirit, being treated in a similar way, and then put into 95 per cent. alcohol for twenty to thirty minutes. The best way is to take one section right through to 95 per cent. alcohol, examine it under the microscope and use it as a guide for staining the remainder. The length of time the sections are left in the 95 per cent. alcohol decides the blueness and clearness of the nuclei. If this method is followed out the nuclei show up as sharp as when eosin is used as counter-stain.

(Workers using paraffin sections can get the same results if they have the sections under absolute alcohol about the same time before they are cleared with xylol.)

When the sections have been in 95 per cent. alcohol the required time they are transferred to carbol xylol; when they appear clear they are put into xylol, but if they should make the xylol cloudy they must be put back into carbol-xylol for a short time. When dealing with a large number of sections it is advisable to have a second dish of carbol-xylol into which the sections

are put one at a time and from this into xylol. They are then mounted in "acid balsam," that is, xylol balsam saturated with salicylic acid crystals.

Résumé

(1) Brains and cords to be fixed in formol saline 2-10 days.
(2) After one week in formalin the brain is sliced.
(3) Cut pieces of tissue for embedding.
(4) Equal parts of spirit and water 24 hours.
(5) Spirit 24 hours.
(6) Absolute alcohol 24 hours.
(7) Equal parts absolute alcohol and ether 48 hours, changed once.
(8) 2 per cent. celloidin 7-10 days
(9) 4 per cent. celloidin 4-7 days.
(10) Embed in 8 per cent. celloidin.
(11) When hard enough cut out tissue and mount on wooden blocks.
(12) Cut sections 6-10μ and place in spirit as cut.
(13) Transfer to distilled water for a few minutes.
(14) Transfer to iron hæmatoxylin ; time according to stain used.
(15) Wash in tap water ; change immediately and leave until sections are blue, if necessary differentiate in acid alcohol.
(16) Counterstain in Van Gieson stain 5-10 seconds.
(17) Quick wash in distilled water.
(18) Spirit a few seconds.
(19) 95 per cent. alcohol 20-30 minutes.

(20) Carbol xylol ; xylol ; mount in acid balsam.

N.B.—The times which have been given are for large pieces and numbers, but when pieces are done singly and are of small size the time may be cut down.

Staining Celloidin Sections in Sequence and Serially

Sometimes it is important that a section of each segment of the cord from the first cervical to the fifth sacral should be cut, stained and mounted in a known order. This procedure is known as staining in sequence, and is time-devouring no matter what method is used. If the worker carried out this work in its simplest form, namely, cutting a section, staining, mounting, labelling and numbering it, before he cuts another it would be a very slow process. The following details are given of a method for staining the cord in sequence which will save a great deal of time.

Each block is arranged in order according to its segment ; then several sections are cut from each block and placed either in small bottles or in watch glasses containing spirit and bearing the number corresponding to the block. Keeping several sections from each block allows for loss by damage, or for an extra set if required, and may save time and trouble later. When the sections are all cut, the worker can start staining.

He must have water, stain, dehydrating and clearing fluids ready for use.

The following list of dishes needed is given before detailing the method proper. As the sections are in spirit the first dish must contain water, the second hæmatoxylin, the third and fourth tap water, the fifth counter-stain, the sixth distilled water, the seventh spirit, the eighth 95 per cent. alcohol, the ninth carbol-xylol and the last xylol. After laying the dishes down in the above order a start is made to stain the sections.

The first section is transferred from spirit to water for a few seconds and then into hæmatoxylin to stain. The second section is now transferred from spirit to water. When the first section has been in hæmatoxylin long enough, it is transferred into the first dish of tap water and immediately placed in the second dish of tap water. Returning to the second section it is placed in the hæmatoxylin, then a third section is taken from the spirit and placed in water. By this time No. 1 is ready for counter-staining and is placed in the counter-stain five to seven seconds, washed quickly in distilled water and a few seconds in spirit and left in 95 per cent. alcohol. No. 2 section is now ready to be taken from the hæmatoxylin, so it is washed quickly in the first dish of water and passed into the second one. No. 3 section is then placed in the hæmatoxylin. A fourth section is now transferred from spirit to water.

The first section is transferred to carbol-xylol. No. 2 section is counter-stained and carried as far as 95 per cent. alcohol. No. 3 is taken from the hæmatoxylin and taken through to the second water bath. No. 4 is put into hæmatoxylin and No. 5 is taken from spirit and put into water. The first section is transferred into the dish of xylol, which should be large enough to allow a slide inside. The section is floated on the slide, smoothed out flat with a camel-hair brush, and mounted in acid balsam, and " No. 1 " marked on the slide with indian ink. This completes a cycle, and the succeeding sections should be treated in the same way till all are stained, mounted and numbered. A trial of this method will convince the worker of its usefulness, simplicity and rapidity. The numbering of the section so far is only temporary, so when the balsam has had time to set, the slides are cleaned, properly labelled and named.

Certain segments of the cord have distinct features and are easy to recognise. The first cervical has a peculiar picture of its own ; the anterior and posterior horns resemble a pair of crossed scimitars. The first dorsal has the appearance of a child's first effort to make a capital H. The remaining segments of the dorsal region are not easy to distinguish microscopically, therefore the worker must be very careful to keep them in correct order. The lumbar and sacral regions are not difficult to learn if the worker

bears in mind the size and shape of the horns. The peculiarities mentioned are more noticeable after the sections have been stained.

Serial Sections

Cutting serial sections means cutting them from one piece of tissue and staining them in order as cut, but it may be only necessary to stain every fifth or tenth section. If the worker wishes to stain serial sections of the whole cord, or even of a segment, he will find it inconvenient to have the requisite number of bottles or dishes to hold each separately. To overcome this the section when cut should be taken off the knife with a small oblong piece of toilet paper and placed in a glass dish deep enough to hold all the sections one on top of the other. This is done in the following manner.

A large number of pieces of the paper are cut beforehand, and as a section is cut it is spread out flat on the knife with a camel-hair brush; the piece of paper is then laid over it, pressed gently down with the brush, and drawn, not lifted, off the knife. The section will adhere to the underside of the paper, which is turned and laid in the dish with the section uppermost. When the staining is done the procedure is exactly the same as staining in sequence. Should the worker decide to take only every fifth or tenth section the following plan should be carried out:

In the case of every fifth section five dishes are necessary, of every tenth, ten dishes. Taking as an example the case of staining every fifth section. Five dishes are numbered 1-5; the first section cut is placed in No. 1 dish, the second in No. 2 and so on till the sixth section which is placed in No. 1 dish on top of the previous section. This is carried on till the block is completely cut. By this method each dish will contain a complete set of every fifth section; thus, should one set be damaged, another set is at hand ready to be stained. The pile of sections in each dish must be kept well moistened with spirit.

Résumé

(1) Cut sections 6-10μ, place in spirit in proper order and number.
(2) No. 1 section is transferred to water for a few seconds.
(3) No. 1 section is placed in hæmatoxylin.
(4) No. 2 section is transferred to water.
(5) When No. 1 section has stained long enough, transfer to tap water changing into fresh tap water immediately.
(6) No. 2 section is transferred from water to hæmatoxylin.
(7) No. 3 section is placed in water.
(8) Counter-stain No. 1 section 5-7 seconds.
(9) Quick wash in distilled water.
(10) Quick wash in spirit.
(11) Place in 95 per cent. alcohol and leave for a time.

(12) Take No. 2 section from hæmatoxylin and wash in water.
(13) No. 3 section is now placed in hæmatoxylin.
(14) Transfer No. 4 to water.
(15) Remove No. 1 from 95 per cent. alcohol and place in carbol xylol.
(16) Counter-stain No. 2 and pass along to 95 per cent. alcohol.
(17) Transfer No 3 from hæmatoxylin to water.
(18) Place No 4 in hæmatoxylin
(19) No. 5 is now placed in water
(20) No. 1 is transferred to xylol, mounted in acid balsam and numbered

The cycle is now complete, and as each section is mounted and numbered the others are advanced to the various dishes until all have been stained, mounted and numbered.

Staining for Nissl Bodies

Nissl bodies can be stained in frozen, paraffin or celloidin sections, the last being the most commonly used. If serial sections are needed, then it is advisable to use paraffin. For a quick diagnosis frozen sections may be satisfactory. Various methods of procedure for the staining of Nissl bodies are given in text-books. The following has proved to be most useful and to give constant results.

Celloidin sections are cut at 10μ, and transferred

to spirit as cut. When the sections are all cut they are placed in equal parts of alcohol and ether in order to dissolve out the celloidin ; it is necessary to leave them over-night when the sections are large and numerous, but if only one or two sections are being treated one to two hours will suffice. They should never be left more than sixteen hours in alcohol and ether as they tend to become brittle, causing difficulty in mounting whole sections. After being in alcohol and ether they are transferred back to spirit two to three minutes, then placed in distilled water for about an hour. This is not absolutely necessary, but it helps to bring out the violet tint in the final picture. When the sections are transferred from the spirit to water they tend to dart about on the surface ; this can be guarded against by holding the section underneath for a few seconds. Great care must be taken when doing this, otherwise a part of the section may jump to the surface and break away from the rest of the section. The sections should be allowed a certain amount of freedom, because it helps them to spread out flat, which is beneficial for staining.

After washing in distilled water they are stained in 1 per cent. watery solution of Grübler's toluidin blue ; this stain has given better results in the hands of the author than any other dye. The sections are placed in the stain and put into the paraffin oven at about 50° C. for ten to twenty minutes. They are then transferred to water

(not necessarily distilled water) and differentiated. The differentiating fluids used are Gothard's solution and absolute alcohol, as they are the best and quickest. The sections, taken one at a time, are placed in a dish of Gothard's solution for a few seconds, moved backwards and forwards in it with the glass hook, and are then transferred to absolute alcohol for a few seconds ; after agitation in this way they are passed into a dish of clean xylol. The section should now be examined under the microscope ; if differentiation has not gone far enough the section is placed back into Gothard's solution and the processes repeated until the desired picture is obtained. The worker must remember that Gothard's solution differentiates quickly, therefore he must be careful not to leave sections too long in it. Once the sections have reached the point of differentiation desired they are mounted from xylol, either in Mersol or thick cedarwood oil ; in these mountants the colour of the Nissl granules is retained indefinitely. Often, in fact usually, sections pale rapidly if mounted in balsam.

Paraffin sections are differentiated in a similar way, but being already on slides the solutions are poured on from drop bottles, it being understood that the paraffin has been removed previous to their staining. The worker will no doubt find that with a little experience he will be able to handle a large number of sections or slides at the same time. A point in regard to paraffin

sections should be mentioned. As the stain comes out quicker in paraffin than in celloidin sections it is often not necessary to use Gothard's solution, absolute alcohol being sufficient to differentiate.

When staining frozen sections the worker must remember that strong alcohols make them shrink and cling in folds which are difficult to eliminate once they are in. The best way is to pass them through several graded strengths of alcohols, otherwise the procedure is similar to that for celloidin. Very little has been said about frozen sections in this chapter, because they are seldom used for the staining of Nissl bodies.

As tissue from the central nervous system has a great tendency to shrink in paraffin work, a slightly modified method of infiltration has been adopted. Paraffin with a melting-point about 50° C. is taken, and enough xylol poured on to it to make a mixture which will keep in solution in the incubator at 37° C. The tissue is dehydrated and cleared and placed in this mixture in the incubator at 37° C. from two to three days, then passed through the paraffin oven more quickly than normally. By this method the tissue does not shrink so much.

Résumé (*Celloidin sections*)

(1) Cut sections at 10μ and place in spirit as cut.
(2) Equal parts of alcohol and ether for not more than 16 hours.

(3) Wash in spirit 2-3 minutes.
(4) Wash in distilled water 1 hour.
(5) Stain in 1 per cent. toluidin blue 10-20 minutes in paraffin oven, at 50° C.
(6) Transfer to water.
(7) Place in Gothard's solution a few seconds, agitate.
(8) Transfer to absolute alcohol a few seconds, agitate.
(9) Place in clean xylol (7, 8, 9, repeated till picture is obtained).
(10) Mount in Mersol or thick cedarwood oil.

N.B.—When differentiating, the alcohol should be changed when it has taken up a distinct blue colour.

Staining Sections of the Cord in Sequence for Nissl Bodies

Sections for Nissl staining in sequence may be taken from the stock of sections which were cut for hæmatoxylin staining in sequence. To stain for Nissl bodies by the same method would require a longer time, therefore the routine of staining has been varied. Twelve small wide-mouthed bottles are required: six for alcohol and ether and six for toluidin blue. A label marked off into six squares is stuck on each bottle. The method proceeds thus:—

No. 1 section is placed in one of the bottles containing alcohol and ether and numbered 1

in the first square on the label. Sections up to No. 6 are placed in the remaining five bottles of alcohol and ether, each bottle being numbered as a section is placed in it. This makes six bottles, each containing a section, numbered 1-6 in the first square of the labels. As there is only one section in each bottle it will not take long for the celloidin to be dissolved, ten to fifteen minutes being usually long enough. During this time of waiting the other six bottles are washed out with distilled water and the toluidin blue filtered in, each bottle being about half filled. They are then numbered in the first squares of the labels 1-6.

When the sections have been long enough in alcohol and ether they are transferred to the corresponding bottles of stain. This is done in the following way:—No. 1 section is transferred into spirit, then into distilled water and from water into the stain, which is placed in the paraffin oven at about 50° C. for ten to twenty minutes; each section is treated in this manner until the first six sections are in the bottles of stain and in the paraffin oven. The bottles of alcohol and ether are now reloaded with sections 7 to 12, the numbers being placed in the second square of the labels. The time of putting the first bottle and section in the paraffin oven should be taken note of, and when it has been in long enough it is taken from the stain and placed in water.

From this point only one section at a time is

dealt with. The section in water is differentiated, cleared and mounted exactly in the manner already described for the staining of Nissl bodies. When the section is mounted, the slide is numbered temporarily according to its order. After this is done, No. 7 section is taken from alcohol and ether, passed through spirit, then water, placed in the bottle of stain from which No. 1 section was taken and the label marked 7. No. 13 is then transferred to alcohol and ether and the label numbered 13. It will be seen by this time that the number on each bottle advances by six, therefore the worker should have no difficulty in keeping the sections in their order. The bottle of stain which has No. 7 section in it is placed in the paraffin oven and No. 2 is brought out, and the section is treated in the same way as No. 1, and the cycle keeps on moving round until all the sections are stained, mounted and numbered in their correct order.

When sections have been mounted in Mersol or cedarwood oil, a sticky substance is apt to spread over the slide ; to remove this satisfactorily the following details should be attended to :—Once mounted, the slide is placed in the incubator twenty-four hours, taken out and left on the bench for twenty-four hours. By this time the cover slip has set. Each slide is then dipped into xylol, cleaned with a soft cloth, labelled and numbered.

Résumé (*Nissl staining of cord segments in sequence*)

(1) Cut sections 10μ and transfer to spirit in order.
(2) Number off 6 small wide-mouthed bottles 1-6.
(3) Pour equal parts of alcohol and ether into each bottle.
(4) Transfer first 6 sections, one in each bottle 1-6, 10-15 minutes.
(5) Number 6 more bottles 1-6.
(6) Filter 1 per cent. toluidin blue into each bottle, half full.
(7) Remove No. 1 section from alcohol and ether to spirit.
(8) Transfer to distilled water.
(9) Put section into stain in bottle No. 1.
(10) Place in paraffin oven 10-15 minutes, noting the time of entering.
(11) Treat remaining five sections which are in alcohol and ether the same as No. 1, placing them in corresponding bottle of stain.
(12) Reload alcohol and ether bottles 7-12.
(13) Take No. 1 from paraffin oven when stained long enough.
(14) Transfer section into water.
(15) Differentiate as described in Nissl body staining.
(16) Mount in cedarwood oil or Mersol.
(17) Transfer No. 7 section to stain in the same way as No. 1.

(18) Place No. 13 section in alcohol and ether and number the bottle 13.
(19) Treat No. 2 section as No. 1 was treated.
(20) Keep the cycle moving till all sections are stained, mounted and numbered.

The following is another method by which sections may be stained and mounted in recognised order, but they will not be in proper sequence. This method has the advantage of being more economical, both in time and material used, also taking up less room in storage. The idea is to get sections which can be easily recognised. It is essential that the cord should be complete, and also that the worker should learn that the cord is comprised of eight cervical, twelve dorsal or thoracic, five lumbar and five sacral segments.

Six wide-mouthed bottles are needed and on these are stuck fairly large labels numbered in one corner 1-6. The bottles are three parts filled with spirit, and the sections as cut are put into them in the order given in the following description :—

No. 1 bottle	*No. 2 bottle*	*No. 3 bottle*
1st cervical	2nd cervical	3rd cervical
7th cervical	8th cervical	3rd dorsal
1st dorsal	2nd dorsal	9th dorsal
7th dorsal	8th dorsal	2nd lumbar
1st lumbar	1st sacral	2nd sacral

No. 4 bottle	*No. 5 bottle*	*No. 6 bottle.*
4th cervical	5th cervical	6th cervical
4th dorsal	5th dorsal	6th dorsal
10th dorsal	11th dorsal	12th dorsal
3rd lumbar	4th lumbar	5th lumbar
3rd sacral	4th sacral	5th sacral

When the sections have been cut and placed in their respective bottles a record should be kept of what each bottle contains. The worker can either stain the section with hæmatoxylin or for Nissl bodies. In either case the five sections in each bottle are treated at the same time and are mounted on one slide in the order given. A trial of this method will prove its simplicity.

NEUROGLIA

Mallory's Neuroglia Method

Mallory's method for neuroglia may be used either with celloidin or paraffin sections; the following is that for celloidin sections. The tissue is fixed in Zenker's fluid, and should be put into the fixative as soon after death as possible. Mallory states that if twenty-four hours elapses before this is done the results are negative in the majority of cases, and if any results are gained at all they are very poor.

The tissue after being in Zenker's fluid twenty-four hours is transferred to 80 per cent. alcohol for

twenty-four hours; it is then embedded in celloidin in the usual way. Sections are placed in Gram's iodine for ten to fifteen minutes; they are then passed through several changes of 95 per cent. alcohol to remove the iodine. Next they are washed in water and transferred to 0·25 per cent. potassium permanganate for three to ten minutes. After washing in water they are placed in 5 per cent. oxalic acid for five to ten minutes or longer.

The sections are now thoroughly washed in several changes of water, and stained in phosphotungstic acid hæmatoxylin for twelve to twenty-four hours. After staining, the sections are taken straight into 95 per cent. alcohol for not more than one to two minutes, as the alcohol extracts the reddish colour and destroys the sharp contrast between the different fibrils. If the worker wishes to get rid of the reddish stained structures altogether, the sections are placed in a 10 per cent. solution of chloride of iron for one to several minutes and then washed thoroughly in water. Section smust be cleared in xylol, and this is done by the filter paper blotting method; other clearing fluids make the colour fade.

Résumé

(1) Fix in Zenker's fluid for 24 hours.
(2) Wash in running water 24 hours.
(3) Transfer to 80 per cent. alcohol and leave for 24 hours.

(4) Embed in celloidin in the usual way; cut sections at 6-8μ.
(5) Transfer to Gram's iodine 5-10 minutes.
(6) Wash sections in several changes of 95 per cent. alcohol.
(7) Wash in water and place in 0·25 per cent. potassium permanganate 3-20 minutes.
(8) Wash in water and transfer to 5 per cent. oxalic acid 3-10 minutes or longer.
(9) Wash thoroughly in several changes of water.
(10) Stain 12-24 hours in phosphotungstic acid hæmatoxylin.
(11) Put sections straight into 95 per cent. alcohol 1-2 minutes.
(12) Clear with xylol by the blotting method. Mount in xylol balsam.

We have found this method applicable to tissue fixed in formol saline, although the outline of the fibres is not quite so sharp. If Zenker fixed tissue is cut in celloidin it stains the knife badly and this fixative is therefore usually avoided in celloidin work. If, however, the sections are passed into 5 per cent. mercuric chloride after cutting, and are then given a longer time in strong Lugol's iodine, good results are obtained. Quite good results are even got with certain tissues by using strong iodine without the use of mercury.

We have not found that carbol-xylol causes the colours to fade.

Mallory's Anilin Blue-Orange G. Stain (Modified) for Neuroglia Fibres and Cells

The following method is a slight variation of Mallory's Anilin Blue-Orange G. method and may be used on paraffin or celloidin sections.

Tissue to be embedded in paraffin is best fixed in Bouin's fluid direct, but if it has already been fixed in formol saline it should be put into Bouin's fluid for sixteen to twenty-four hours. It is then passed through the alcohols and paraffin in the usual way. When sections are cut from Bouin fixed material they are definitely yellow, but this does not interfere when staining for neuroglia, as the mordant, used before the stain, removes the yellow colour altogether ; but, if any of the sections are to be stained with hæmatoxylin and a counter-stain, it is best to place them in a dish of warm water, which removes the colour very quickly. If the picric acid is not washed out of the sections it interferes with good staining by the hæmatoxylin.

Tissue for celloidin infiltration may be fixed in the ordinary way in formol saline.

Celloidin Sections.—Celloidin sections are cut at 8-10μ, and washed in distilled water. They are then placed in the iron-alum mordant (*see* Résumé) for ten to thirty minutes, after which they are washed in distilled water and stained for one to two minutes in acetic acid fuchsin ($\frac{1}{2}$ per cent. of each).

They are now washed well in distilled water and put into 4 per cent. phosphomolybdic acid for thirty to forty-five minutes. They are again washed in distilled water and stained in Mallory's Anilin Blue-Orange G. for fifteen minutes. The sections are washed rapidly in distilled water and differentiated in 95 per cent. alcohol. When differentiated they are mounted and cleared by the blotting method ; this consists in blotting and pouring on xylol and repeating the process until the sections are clear. They are then mounted in acid balsam.

Carbol xylol must not be used for clearing in this method or the colours will fade.

Paraffin Sections.—Sections are cut at 6-8μ and mounted on clean slides. When the sections are dry the paraffin is dissolved off with xylol ; the sections are then washed with absolute alcohol, then spirit, and thoroughly washed in distilled water. Further procedure is the same as for celloidin sections, except that the differentiation is done with absolute alcohol.

The mordant and the stains may be used repeatedly, but the phosphomolybdic acid should be thrown away after use.

Résumé

(1) Mordant 10-30 minutes in iron alum 1 gm. ; sulphuric acid 1 c.c. ; 50 per cent. alcohol 98 c.c.

(2) Wash in distilled water.
(3) Stain for 1-2 minutes in ½ per cent. acid fuchsin dissolved in ½ per cent glacial acetic acid.
(4) Wash well in distilled water.
(5) Put into 4 per cent. phosphomolybdic acid for 30-45 minutes.
(6) Wash well in distilled water.
(7) Stain for 15 minutes in water soluble anilin blue 0·5 gm. ; Orange G. 2 gm. ; oxalic acid, 2 gm. ; aq. dest, 100 c.c.
(8) Differentiate in 95 per cent. alcohol for celloidin sections, or absolute alcohol for paraffin sections.
(9) Clear in xylol (*not* carbol xylol).
(10) Mount in acid balsam.

Results

Glia fibres	Violet
Glia cells	Blue
Axis cylinders	Blue
Myelin	Gold with slight rose tint
Ganglion cells and vessel walls	Dark blue
Nucleoli and blood corpuscles	Bright red
Nuclei	Greenish yellow

Mucicarmine (Mayer)

This stain is used for the locating of mucin, and hæmatoxylin is used as a contrast stain.

Either of the two may be used first with equally good results. It is immaterial whether celloidin or paraffin sections are used ; it is also easy to follow the method and accomplish the end in view. It does not need any special fixative, as formol fixed tissue has always given satisfactory results. Sections are cut in the usual way, washed in distilled water and stained.

When the hæmatoxylin is used first, the sections are stained as though they were to be counterstained with " van Gieson," but instead of this they are put into mucicarmine diluted 1 in 5 for one hour in the paraffin oven at about 50°C. They are then washed well in distilled water, dehydrated, cleared and mounted in xylol balsam. When mucicarmine is used first it is simply the above method reversed, excepting that the staining in hæmatoxylin should not be so long.

Résumé

(1) Cut sections 8-10μ and wash in distilled water.
(2) Stain in mucicarmine, diluted 1-5 for 1 hour in the paraffin oven at about 50°C.
(3) Wash thoroughly in distilled water.
(4) Counter-stain in hæmatoxylin.
(5) Wash well in tap water.
(6) Dehydrate, clear and mount in xylol balsam.

Myelin Sheaths

Tissue to be used for the staining of myelin sheaths is fixed in formol saline in the same

manner as tissue for ordinary celloidin. Several methods have been devised for staining myelin sheaths in ordinary celloidin sections, but none has been fully satisfactory ; therefore the pieces have to be mordanted in special fluids before they are embedded in celloidin. This is done as follows :—

Pieces are selected and placed in the mordant for a time, which varies according to the size, thickness and number of pieces. Tissue may be mordanted in Muller's fluid, Weigert's primary mordant, or in the latter with the addition of calcium hypochlorite. Muller's fluid is a very slow mordant necessitating immersion for several months. Weigert's primary mordant is much quicker, seven to ten days being usually sufficient. Weigert's mordant containing calcium hypochlorite is the most rapid, and its action can be further hastened by putting it into the incubator. By this method two to four days will suffice for mordanting. Good mordanting is essential, otherwise it will be impossible to get proper staining. When the tissue has been mordanted it is passed through the alcohols and embedded in celloidin. Each time the pieces are changed from the different alcohols they should be shaken in the bottle to allow of the sediment being removed; if this is done the celloidin is not contaminated with chrome salts and may be used repeatedly.

The bottles used for holding the pieces of tissue should be wide enough to allow them to lie flat.

When embedding in the 8 per cent. celloidin it is advisable to cover the bottom of the dish with tinfoil, otherwise the tissue may adhere to the glass and be broken on removal. When the celloidin mass is hard enough the pieces of tissue are cut and mounted on wooden blocks; they may be preserved indefinitely in spirit. At no time previous to staining should tissue or sections which have been in chrome be left in anything weaker than spirit.

Section Cutting.—Great care and precaution must be taken in cutting mordanted sections as they differ materially from ordinary celloidin sections. They are usually cut at 20μ, sometimes thicker. After cutting a section it is spread out flat on the knife, always keeping the knife-block and sections wet with spirit. An oblong piece of thin paper, broad enough to cover the section, is laid on it and smoothed over with a soft brush. It is then drawn off the knife. The section should remain on the underside of the paper, which is now placed in a dish with the section uppermost.

The sections are all cut in this manner and placed in their correct order in the dish, being kept just moist with spirit, as too much may cause them to float off the paper. The best paper to use is white toilet paper.

"*Plating*" *Sections.*—Once the sections are all

cut they are plated as follows :—Take a plate of glass, or preferably " opalite," clean it well with soap and water and dry it. After it is dry coat it with Obregia's solution. It is best to pour the solution on the middle of the plate and spread it out evenly with the fingers, rubbing it in well. The plate is now put in a warm place to dry, in the incubator or on the top of the paraffin oven. It is allowed to dry until the surface is hard and smooth.

If there are any bare spots on the plate after the gum has dried, the worker must either wash the plate and make it afresh or carefully avoid the bare spot when laying the sections on the plate. The sections are taken from the dish one at a time by taking hold of the paper at one end ; the paper is turned over with the section face downwards on to the dried gummed plate and smoothed with the brush. The paper is now lifted off and the section should be left on the plate. In order to keep the section moist, gently replace the paper in such a way as not to interfere with the position of the section following. Let the first paper overlap the plate as well as the section ; when the second section is on the plate let the second paper overlap the first one and so on to the bottom of the row.

As one row is completed the worker should pour spirit on it to ensure that the paper and the sections are kept moist. When the sections are all on the plate the papers can be removed by

taking hold of the overlapping ends and carefully lifting them off. The whole plate of sections is then blotted down firmly and carefully. A very thin solution of celloidin is now poured on the plate, being sure that every section is covered. The excess of celloidin is poured off, leaving enough behind to ensure a thin even film. The plate is placed on an absolutely level bench and left until the celloidin becomes hard enough to allow the finger to be moved over it without lifting up the celloidin. The whole plate is then flooded with spirit for a few minutes.

Next take a sharp knife and cut all round the edges as close to the sections as is consistent with safety, then cut between the sections, singly or in strips as desired. Pour off the spirit and place the plate in warm water; the gum will dissolve and the sections will float off either singly or in strips according as they were cut on the plate. The sections may be preserved in spirit until they are wanted for staining purposes. If the sections are plated in a methodical manner the work of staining a series may be much simplified. By this method sections in sequence of the cord may be stained and mounted on one or more slides according to the strips cut. It will be found that this method is much quicker and easier than cutting the sections singly or in strips of no order at all.

Staining.—Sections to be stained may be placed

in "Gliabeize" for half an hour at room temperature, but this is not essential, though it does sometimes seem to give a better colour to the sections. They are then washed in water and stained in hæmatoxylin made according to Kultschitsky's formula or Anderson's modification of it.

If Gliabeize is omitted, the sections are passed from spirit into water and from this straight into the stain. The sections are left in the stain for twenty-four to forty-eight hours, preferably the latter, in the incubator at 37° C. or for twelve to twenty-four hours in the paraffin oven at 50° C.; they are then transferred without washing into Muller's fluid for ten to fifteen minutes, washed in several changes of tap water and differentiated with $\frac{1}{4}$ per cent. potassium permanganate and Pal's solution.

Differentiation.—The sections are transferred from tap to distilled water. The sections are now treated with potassium permanganate and Pal's solution alternately. The permanganate bath should never exceed thirty seconds, but the sections may be left in Pal's solution longer. The best plan for the worker to follow is to make the first permanganate bath a few seconds only, so that he may ascertain the intensity of the staining. If the stain does not show signs of coming out quickly the time can be lengthened. The two baths mentioned are repeated until the sections have been

differentiated to the desired stage. If the Pal's solution becomes brownish it must be thrown away and a fresh lot used, also when a large number of sections have been stained it will be necessary to change the permanganate once or twice. Attention to these points will ensure a black and white contrast, instead of a dirty brown.

If the work of differentiation has to be interrupted before it is finished, the section may be left in Pal's solution or in distilled water until the differentiation can be completed. The best way of performing the differentiating is to pour the liquid out of the dish, leaving the sections behind, and then to pour the other liquid into the dish containing the sections. By doing this there is no fear of the sections becoming damaged. Three dishes are needed, two for the solution and one in which the sections stay until they are finished. When the desired picture has been obtained, the sections are put into distilled water for about an hour, the water being changed once during that time. They are then put into tap water for several hours, after which they are put into spirit; in this they can be left indefinitely if kept in a well-stoppered bottle.

Mounting Sections.—To mount them they are left in spirit for ten minutes, then transferred into 95 per cent. alcohol for twenty to thirty minutes,

then passed into carbol-xylol and then into clean xylol. When clear they are mounted in thick xylol balsam. The times mentioned in this procedure are for a large number of sections ; if only a few have been stained, the dehydrating and clearing will be much shorter.

Counter-Staining with Alum-Carmine.—Transfer sections to be counter-stained from water into alum carmine and place in the paraffin oven at about 50° C. for two to three hours. The sections are then washed in distilled water until the celloidin is a faint pink colour, after which they are dehydrated, cleared and mounted in ordinary xylol balsam.

Résumé

(1) Mordant tissue in Weigert's primary mordant for 7-10 days at laboratory temperature.
(2) Pour off the mordant and place in spirit for 24 hours.
(3) Absolute alcohol 24 hours.
(4) Equal parts of alcohol and ether 48 hours, changing once.
(5) 2 per cent. celloidin 7-10 days.
(6) 4 per cent. celloidin 4-7 days.
(7) Embed in 8 per cent. celloidin with tinfoil on the bottom of the dish.
(8) Mount on wooden blocks ; store in spirit.
(9) Cut sections 20μ and plate as described in detail.
(10) Gliabeize 30 minutes. This step is not essential.

(11) Wash in water and stain 24-48 hours in Kultshitsky hæmatoxylin, or Anderson's modification of it, in the incubator at 37°C., or for 12-24 hours at 50°C.
(12) Without washing transfer to Muller's fluid 10-15 minutes.
(13) Wash well in several changes of tap water
(14) Differentiate as described in detail.
(15) Wash in distilled water for about an hour.
(16) Transfer to tap water for several hours.
(17) Counter-stain if desired.
(18) Dehydrate, clear and mount in xylol balsam.

Weigert's Method

Pieces of tissue are mordanted in Weigert's primary mordant four to six days; they are then taken through the alcohol and celloidin in a similar way to that already described. When the sections are cut they are placed in Gliabeize for twenty-four hours in the incubator at 37°C.; sections may be plated if desired. After the Gliabeize, sections are stained in lithium hæmatoxylin for twenty-four hours, washed in water for thirty minutes or longer and differentiated in Weigert's differentiator until the grey substance appears yellow. The control of the differentiation must be done under the microscope. After proper differentiation the sections are washed thoroughly in water, dehydrated, cleared and mounted in the usual way.

Résumé

(1) Pieces 3-5 mm. thick 4-6 days in Weigert's primary mordant.
(2) Transfer to spirit 24 hours.
(3) Absolute alcohol 24 hours.
(4) Equal parts of alcohol and ether 24-48 hours.
(5) Pass through the celloidin and mount on wooden blocks as already described.
(6) Cut sections 15-20μ; plate if desired.
(7) Transfer to Gliabeize for 24 hours at 37° C.
(8) Wash and stain in lithium hæmatoxylin for 24 hours.
(9) Wash in water for 30 minutes or longer.
(10) Differentiate by method described in detail.
(11) Wash thoroughly, dehydrate, clear and mount in balsam.

N.B.—According to Hall and Herxheimer the dehydration should be done in the dark. We have found this unnecessary.

Pal's Modification

This method has been almost entirely replaced by the Kultschitsky-Pal method, therefore only a résumé of it will be given.

(1) Harden in potassium bichromate, or preferably in Muller's fluid 3 months or longer.
(2) Pass through the alcohols, celloidin and embed.
(3) Cut sections and stain in Weigert's lithium hæmatoxylin 24 hours.

(4) Wash in tap water until sections are a dark blue colour.
(5) Transfer to 0·25 per cent. potassium permanganate 20-30 seconds.
(6) Wash in water.
(7) Transfer to Pal's solution until sections show the grey substance colourless and the white substance blue.
(8) Wash thoroughly in water.
(9) Dehydrate, clear and mount as usual.

Osmic Acid for Staining Myelinated Fibres (Heller-Robertson)

This method is not often used in the National Hospital laboratory because good results are usually obtained by the myelin method previously explained. Also osmic acid is very expensive, therefore it is not recommended for routine work. Occasionally, when working with nerves, the fibres are not stained by the usual method. This means that the mordant for some reason or other has failed to act. In such a circumstance the following method is advised.

Fresh sections are cut and stained by " Robertson's modification of Heller's method for staining peripheral nerves."

(1) Place the section in Gliabeize for 24 hours.
(2) Wash well in water and stain in 1 per cent. osmic acid for 30 minutes in the dark.

(3) Wash thoroughly in distilled water and place in 5 per cent. pyrogallic acid for 30 minutes.
(4) Wash again thoroughly in distilled water and differentiate by the method given for myelin sheaths (permanganate and Pal's solution).
(5) Wash well in water, dehydrate, clear and mount in xylol balsam.

Loyez Method for Staining Myelin Sheaths in Ordinary Celloidin or Paraffin Sections.

The following, which is a slight modification of Loyez method, has in the hands of the author given well-stained sections with sharp contrasts between the blue myelin sheaths and the colourless background. The only disadvantage, as compared with the Weigert Pal method, is that nuclei are deeply stained.

Sections are cut from ordinary celloidinised tissue at 15-20μ, washed in distilled water and placed in 4 per cent. iron alum for twenty-four hours at laboratory temperature. The sections are then washed thoroughly in distilled water and stained, for two to four hours in the paraffin oven at about 50° C., in the following hæmatoxylin solution, which must be made up immediately before use from unripened hæmatoxylin:—

Dissolve hæmatoxylin 1 gramme in 10 c.c. of absolute alcohol. Add 90 c.c. of distilled water and then 2 c.c. of a saturated solution of lithium

carbonate. The sections after removal from the stain are thoroughly washed in tap water and differentiated in acid alcohol.

The sections are dehydrated and cleared in the usual way and mounted in ordinary balsam.

Résumé

(1) Cut section at 15-20.
(2) Wash in distilled water.
(3) Transfer to 4 per cent. iron alum 24 hours at laboratory temperature.
(4) Wash thoroughly in distilled water.
(5) Stain in Loyez hæmatoxylin for 2-4 hours at about 50° C.
(6) Wash thoroughly in tap water.
(7) Differentiate in acid alcohol.
(8) Transfer to clear tap water.
(9) Dehydrate and clear in the usual way.
(10) Mount in ordinary balsam.

Osmic Acid

Osmic acid is used for the detection of early degeneration of the myelin. Tissue stained by osmic acid is usually embedded in celloidin, the mordanting and staining being done before the tissue is passed through the alcohols and celloidin ; therefore the sections on cutting are ready to be mounted without further staining. The two methods in most common use are Marchi's method and Busch's modification of it. Muller's

fluid is the mordant used in both methods, but they differ slightly in the staining mixture. The procedure is very slow and takes a considerable time to accomplish, and failure may result owing to improper infiltration of the osmic acid. Of the two methods mentioned Busch's modification is preferable, in so much as tissue is less friable after the Busch than after the Marchi method.

Marchi

Tissue may be fixed in formalin or hardened in Muller's fluid for at least eight days. Pieces not more than 2 mm. thick are then taken and put into Muller's fluid for ten to fourteen days. They are then placed in freshly made Marchi's fluid for eight days. Should the mixture lose its osmic acid smell it must be renewed. Roussy and Lhermitte say that the bottle containing this fluid should have either fat-free cotton wool or filter paper on the bottom under the sections. After the pieces have been in the staining fluid for the required length of time they are washed in running water for twenty-four hours. They are then passed quickly through the alcohols and celloidin and embedded in the usual way.

Résumé

(1) Select pieces of formol fixed tissue, not more than 2 mm. thick.
(2) Place in Muller's fluid for 10-14 days.

(3) Put in Marchi's fluid for 8 days at 37° C.
(4) Wash in running water for 24 hours.
(5) Pass through the alcohols and celloidin and embed as already advised.
(6) Cut sections at 20μ and mount in Gurr's mounting medium direct from 95 per cent. alcohol.

N.B.—Some workers recommend the addition of either 20 drops of dilute nitric acid or 10 to 20 c.c. of 1 per cent. acetic acid to each 100 c.c. of Marchi's fluid to improve the penetration.

Busch's Modification

Pieces of formol saline fixed tissue not more than 2 mm. thick are placed after washing either for a few hours in water or for twenty-four hours in 2 per cent. potassium iodate in Busch's fluid for seven days at room temperature. The bottle used should be of plain glass with a well-fitting stopper, and should have two or three pieces of filter paper laid on the bottom inside. It should remain in the daylight as much as possible. The tissue is then washed in running water for two or three hours, and may then be transferred to bichromate solution to which calcium hypochlorite has been added (chromo-hypochlorite) for seven days in the incubator at 37° C. After this it is washed in running water for twenty-four hours, dehydrated quickly and passed through the celloidin. When the sections are cut they are transferred from spirit to 90 per cent. alcohol

and mounted directly from this in Gurr's neutral mounting medium.

Résumé

(1) Select pieces of tissue not more than 2 mm. thick.
(2) After washing place in Busch's fluid for 7 days in the daylight as much as possible.
(3) Wash in running water 2-3 hours.
(4) Transfer to chromo-hypochlorite 7 days at 37° C.
(5) Wash in running water for 24 hours.
(6) Spirit 1 hour.
(7) Absolute alcohol 1 hour.
(8) Equal parts of alcohol and ether 2-3 hours.
(9) 2 per cent. celloidin 4 days.
(10) 4 per cent. celloidin 4 days.
(11) Embed and mount in blocks as usual.
(12) Cut sections at 20-30μ and mount from 90 per cent alcohol in Gurr's neutral mounting medium.

N.B.—The after treatment with chromo-hypochlorite solution, which is added here to Busch's method, appears to prevent the fat stained with osmic acid from dissolving out during dehydration in alcohol.

CHAPTER III

FROZEN SECTIONS AND METHODS IN WHICH THEY ARE USED

Frozen sections can be used for practically any method of staining with excellent results, and are even in a few cases better than paraffin or celloidin sections. If it were not for a few disadvantages associated with frozen sections, this work would take the premier place in histology.

A great deal may be written on staining methods which can be adapted to frozen sections, but only those in most common use and suitable to the work are explained in the following pages. The methods given will be for the most part for tissue fixed in formol saline, as in the author's experience this fixative has given uniformly satisfactory results with the usual methods of staining.

There are several different kinds of microtome on the market suitable for cutting frozen sections. CO_2 is usually the medium for freezing tissue. A wedge-shaped knife is the most satisfactory, and the sections are best cut by a direct pull. When cutting frozen sections the worker should have a bottle of gum acacia at hand ; a little of

this is poured on the freezing box and the piece of tissue to be cut is placed on the gum.

When freezing tissue, and especially a small piece, it is unwise to freeze it too quickly as it becomes too hard for cutting, thus tending to blunt the knife. The best way is to allow the gas to escape for a few seconds only, after this turn off the gas for a few seconds ; then feel the top of the tissue ; should it still be soft allow more gas to escape. As soon as the tissue feels hard try the knife on it. With large and thick pieces of tissue the worker will have to allow a longer escape of gas, but a little practice will soon teach him how much freezing is necessary.

The hardness of the tissue governs the method of cutting the section. If the tissue is fairly hard the sections may be flicked into a dish of water placed at a convenient distance by pulling the knife across the tissue quickly. As the tissue gets softer it will be found that the section will push its way up the knife blade ; when this happens the knife should be pulled across more slowly.

The section should be removed from the knife by placing the finger flat on the knife behind it and sweeping the section off on the finger. The finger is dipped into a dish of water, when the section will be found to float off ; when this is done properly a perfect section should result. When the knife is cutting well a large number of sections may be collected on it before they are

taken off, for as the sections are cut they push one another farther up the knife.

After the sections are cut they can either be carried further or stored in 5 per cent. formalin until it is convenient to stain them, as they will keep indefinitely in this solution. Most pieces of tissue can be cut on an ordinary freezing microtome regardless of their size.

It must be remembered that if any piece of tissue overlaps the freezing-box there will be great difficulty in freezing it. This difficulty can be overcome by using a piece of perforated aluminium sheeting ; this should be a trifle larger than the piece of tissue to be cut. A few drops of gum are dropped on the freezing-box, the aluminium plate is then placed in the gum, a little more gum is poured on the plate and the tissue on the top of this. The CO_2 is allowed to escape as already described and the whole will freeze uniformly, resulting in good sections being cut in the usual way.

Frozen sections are particularly suitable for demonstrating the following structures :—Myelin sheaths, neurofibrils, neuroglia, Nissl bodies and fatty degeneration.

Staining Myelin Sheaths in Frozen Sections

Sections are cut at 24-30μ and are transferred to distilled water for five to ten minutes, and then put in Weigert's primary mordant

containing calcium hypochlorite for twenty-four to forty-eight hours in an incubator at 37° C. After this they are placed straight into Weigert's Gliabeize for fifteen to thirty minutes at laboratory temperature. The sections are now washed in plenty of distilled water and transferred to modified Kultschitsky's hæmatoxylin for one hour in a paraffin oven between 45°-55° C. Throughout this procedure a wide-mouthed glass-stoppered bottle is the most suitable receptacle, as the section can be easily handled in it. It is advisable to stain more sections than are needed, as some may be broken during the process of changing from one fluid to the other.

After staining they are transferred, without washing, into Muller's fluid contained in a flat dish for ten minutes at laboratory temperature. They are then washed well in plenty of tap water, changed into distilled water for a few minutes, and differentiated in a similar way to that explained for celloidin sections. Frozen sections are easily differentiated and must not be left in permanganate for more than a few seconds for the best results to be obtained.

The sections are mounted direct from water. Float a section on a clean slide, taking care to straighten out all the folds, then blot firmly but carefully with the best white filter paper, dehydrate, clear in xylol and mount in Canada balsam. A point to be remembered is that the sections after blotting should be allowed almost

to dry before the dehydration is carried out, thus preventing the possibility of the sections floating off the slide. This method for myelin sheath staining is quick, very simple, easy to learn, and when carried out gives consistently good results.

Résumé

(1) Fix in formol saline.
(2) Cut sections on freezing microtome at 24-30μ.
(3) Wash in distilled water 5-10 minutes.
(4) Place in mordant 24-48 hours in the incubator at 37° C.
(5) Transfer to Gliabeize 15-30 minutes at room temperature.
(6) Wash well in plenty of distilled water.
(7) Stain for 1 hour in paraffin oven in modified Kultschitsky's hæmatoxylin.
(8) Without washing place in Muller's fluid 10 minutes.
(9) Wash well in tap water; transfer to distilled water.
(10) Differentiate as described for celloidin sections
(11) Wash well in distilled water and change to tap water.
(12) Float a section on clean slide and blot carefully but firmly.
(13) Dehydrate and clean in xylol.
(14) Mount in Canada balsam.

Neurofibrils

There are many ways of staining neurofibrils, the method in most general use being that of Bielschowsky or Da Fano's modification of this. The latter method as originally described by Da Fano is given in the *Microtomists Vade-mecum*, to which reference may be made. In the following paragraphs the details vary slightly from the original. This method requires practice, strict adherence to detail, and patience. In the experience of the writer, Bielschowsky's original method is excellent for peripheral nerves and Da Fano's modification for brain and spinal cord.

Da Fano's Modification of Bielschowsky's Method

This method is given first because brain and cord are stained for neurofibrils more frequently than peripheral nerves in routine laboratory work.

Sections are cut at 16μ, and after cutting are transferred to distilled water for a few minutes; they are then put into equal parts of methyl alcohol and 20 per cent. chemically pure formalin for twenty-four hours at laboratory temperature. The sections are then washed in several changes of distilled water for an hour or more. After washing they are placed in 2-3 per cent. silver nitrate in a brown bottle for sixteen to twenty-

four hours in the incubator at 37° C. They are then washed quickly (one or two seconds only) in distilled water and placed in Da Fano's ammoniacal silver for half an hour at laboratory temperature.

Experience alone will show the time of washing in distilled water, as too short a time gives too large a deposit of silver, while too long will wash out too much silver. One section at a time is washed by taking it upon a glass hook, submerging it in distilled water, swishing it twice or thrice gently and then placing it in the ammoniacal silver. If a large number of sections are being stained at the same time the water should be changed occasionally. The glass hook used for handling the sections should be washed each time before taking the sections from the silver nitrate.

It is essential that all glass ware used must be chemically clean. The most satisfactory way is to wash them out in running water, then with alcohol, and last of all with distilled water.

Before going further a description of how to make the ammonical silver will be given.

Da Fano's Ammoniacal Silver Solution

Take 5 c.c. of a 20 per cent. silver nitrate solution in a 50 c.c. measure, add two drops of a 40 per cent. sodium hydroxide, when a heavy precipitate will be thrown down; this is dis-

solved by adding ·880 ammonia drop by drop, shaking the measure between each drop. This is essential because no more ammonia must be added than is necessary just to dissolve the precipitate. If this is done carefully the resulting solution should be clear, colourless and free from the smell of ammonia; if it is not, the solution is valueless and should be thrown away and a fresh solution made.

After remaining for half an hour in this solution the sections are given a very rapid wash in distilled water and put into 20 per cent. formalin for two to twenty-four hours, the formalin being changed if it should become cloudy. The washing of the sections at this stage is of great importance, because it has a great deal to do with the success of the results. Again, it is only by experience that the time of washing can be judged.

Attention should be paid to the colour of the sections after they are transferred to the formalin, as this will serve as a guide to the duration of the washing of other sections.

At the end of two hours in formalin one section may be toned and mounted as described in the following paragraph.

A section is taken from the formalin, washed well in distilled water, and toned in a solution of gold chloride. This solution is made by adding 3 c.c. of a 1 per cent. solution of gold chloride to every 10 c.c. of distilled water. The section is left in this until it has lost the brown colour

and become blue-grey; it is then washed quickly in distilled water and fixed in 5 per cent. hyposulphite for at least five minutes. After this it is placed in a dish of clean distilled water, washed well, and then floated on to a clean slide, blotted, dehydrated, cleared and mounted in Canada balsam.

Should microscopical examination of the section show the desired picture, the remaining sections may be proceeded with forthwith; should, however, the section be too dark, it is advisable to leave the remainder in formalin up to twenty-four hours.

Résumé (*Da Fano's modification*)

(1) Cut sections at 16μ.
(2) Wash in distilled water for a few minutes.
(3) Place in equal parts of methyl alcohol and 20 per cent. formalin for 24 hours at laboratory temperature.
(4) Wash for a few hours in changes of distilled water.
(5) Transfer to 2-3 per cent. silver nitrate 16-24 hours in the incubator at 37° C. using a brown bottle.
(6) Wash quickly in distilled water.
(7) Place in ammoniacal silver for half an hour at laboratory temperature.
(8) Wash quickly in two changes of distilled water.
(9) Transfer to 20 per cent. formalin 2-24 hours, changing the formalin if it becomes cloudy.

(10) Wash thoroughly in distilled water.
(11) Tone in gold chloride (3 c.c. of a 1 per cent. solution to every 10 c.c. of distilled water.)
(12) Wash quickly in distilled water.
(13) Fix in 5 per cent. hyposulphite for 5 minutes.
(14) Wash in distilled water.
(15) Float on clean slide, blot, dehydrate, clear in xylol and mount in Canada balsam.

Bielschowsky's Method for Peripheral Nerves

Pieces fixed in formol saline are selected for the cutting of sections and placed in 20 per cent. formalin for eight days. The tissue is then washed well in water and sections are cut at 16μ; these are washed in distilled water and placed in 3 per cent. silver nitrate for twenty-four hours in the incubator at 37° C. or in 5 per cent. silver nitrate for two to three days at laboratory temperature. Then they are washed rapidly in distilled water and placed in Bielschowsky's solution (*see* page 134) for thirty minutes at room temperature. After this the sections are washed very quickly in three changes of distilled water and placed in 20 per cent. formalin for twenty-four hours, changing the formalin after ten minutes. They are then counter-stained in van Gieson's solution for ten seconds, mounted, dehydrated and cleared as described in the previous method.

Résumé

(1) Select pieces of formol fixed tissue and place in 20 per cent. formalin for 8 days.
(2) Wash in water and cut sections at 16μ.
(3) Wash in distilled water and place in 3 per cent. silver nitrate 24 hours in the incubator at 37° C.
(4) Wash quickly in distilled water and transfer to Bielschowsky's solution 30 minutes at room temperature.
(5) Wash rapidly in 3 changes of distilled water.
(6) Place in 20 per cent. formalin 24 hours, changing the formalin once.
(7) Wash thoroughly in distilled water.
(8) Fix in 5 per cent. hyposulphite 5-10 minutes
(9) Wash in distilled water.
(10) Counter-stain with van Gieson.
(11) Float sections on a clean slide and blot.
(12) Dehydrate, clear in xylol and mount in Canada balsam.

Fibrous Neuroglia—Anderson's Victoria Blue Method

The most important point to remember in the staining of neuroglia fibres is the proper fixation of the tissue. Strict attention must be paid to the details given in "fixing the tissue." Once the tissue is well fixed, pieces are taken for cutting into sections. Frozen sections are cut

at 16-20μ in the manner already described and transferred to distilled water to wash for several minutes. The worker should remember that this washing is essential to all frozen sections to remove the gum and excess of formalin.

In this method, as originally published in the *Journal of Pathology and Bacteriology*, it was re-recommended that liq. ferri perchlor. (B.P.) should be used. But this iron solution is rather too strong and renders the sections brittle. In the following description the actual percentage of perchloride of iron is stated, which has been found to yield the best results.

The sections when washed are placed in the neuroglia mordant, which consists of equal parts of Anderson's neuroglia mordant and of 5 per cent. pure ferric chloride, for ten to twenty minutes at laboratory temperature; the sections are washed in distilled water and placed in 0·25 per cent. potassium permanganate for five minutes and transferred straight into Pal's solution until they become perfectly white. Sections should be left in Pal's solution until just before staining. They may remain for twenty-four hours or longer in this solution without any harm.

At first only one section at a time should be taken out and stained, but once proficiency is gained several may be stained at the same time.

A clean slide is albuminised by placing a drop of egg albumen on the slide and rubbing it over the slide with the ball of the thumb; then heat-

ing it in the flame of a bunsen burner. A section is transferred from Pal's solution into a dish of clean distilled water, floated on the slide, smoothed out flat and firmly yet carefully blotted down. The blotting should be done with only the best filter paper, such as "Whatman No. 1."* The same piece of filter paper should not be used too often, because it soon gets wet and dirty. The best way is to take a wad of small filter paper and, as one gets wet, remove it and cast it aside. When the section has been blotted down, boil some Grübler's Victoria blue (1·5 per cent. watery solution) in a test tube and flood the sections with this while still hot. The stain is left on for one minute ; a longer time is not detrimental. Pour the stain back into the test tube and cover the section with concentrated Lugol's iodine, which is left on for one minute and then poured off.

The section is now differentiated with equal parts of aniline oil and xylol. The beauty of the sections depends entirely upon the care with which this is done. The aniline oil and xylol mixture is poured on the section for a few seconds only, during which the slide is rocked from side to side. It is then poured off and the xylol is poured freely over the section.

Before any further differentiation is carried out the section must be perfectly cleared. This is done either by blotting and pouring on xylol

* It is sometimes an advantage to damp the filter paper with alcohol to prevent its adhering to the section.

and repeating it until the section is clear, or by blotting the section and warming it gently over a flame, and then flooding it with xylol. The latter method usually proves effective the first time. Once the section is clear the differentiation may be carried out without fear of overdoing it; the usual procedure being to pour on more aniline oil and xylol for a few more seconds, rocking the slide as before, then washing it well in xylol and examining under the microscope. When perfect differentiation is obtained the section is mounted in xylol balsam.

Rapid clearing of the section invariably leaves it a greenish colour; this can be rectified by leaving aniline oil and xylol on the section for about five minutes previous to its being washed in xylol and mounted.

Résumé

(1) Fix brain and cord as already described.
(2) Cut frozen sections at 16-20μ.
(3) Wash in distilled water 5 minutes.
(4) Transfer to neuroglia mixture, 10 minutes at laboratory temperature.
(5) Wash and transfer to 0.25 per cent. potassium permanganate for 5 minutes.
(6) Without washing place in Pal's solution until sections are white.
(7) Albuminise a slide, and float a section on to it from distilled water.
(8) Smooth out the creases and blot.

(9) Pour on boiling 1·5 per cent. Victoria blue and stain 1 minute.
(10) Remove the stain and pour on iodine, leave 1 minute.
(11) Throw off the iodine and differentiate as described and mount in xylol balsam.

Lhermitte's Victoria Blue Method for Neuroglia

This method gives excellent results, though it is by no means a simple one. Patience and attention to details are essential to obtain good results. Lhermitte advises that tissue should be fixed in formalin for fifteen days or more, the formalin to be changed two or three times during the fixation. Frozen sections are cut at 15-20μ, washed for several minutes in distilled water, and put into a saturated solution of mercuric chloride for two hours at laboratory temperature. Without washing, the sections are transferred to modified Flemming's solution for twenty-four to forty-eight hours.

A slide should now be prepared to take a section for staining. This is done in the following manner:—An ordinary cigarette-paper is cut to the size of the slide and spread over it. Distilled water is poured over the paper, making it adhere to the slide. A section is transferred to distilled water with a glass hook, given a rapid wash and put into another dish of distilled water, from

which it is floated on to the paper on the slide. Place the slide on a tripod and flood the section with Grübler's Victoria blue, 1·5 per cent. watery solution. Heat very carefully till the steam rises; this should be done at least three times. Lhermitte advises using the by-pass flame of a Bunsen burner. After cooling, the stain is poured off and without washing, Lugol's iodine is poured on the section and left there for one minute. The iodine is now poured off and differentiation commenced by using equal parts of aniline oil and xylol, which should be repeated until the section appears clear of precipitate.

At this stage the worker proceeds to get rid of the cigarette-paper. This is done by taking hold of one of its corners and turning it completely over on to a clean slide; the paper is carefully lifted off and the section should be left on the slide. If the worker does not succeed the first time he should take another clean slide and make another attempt. When the paper has been eliminated, the differentiation is completed with the aniline oil and xylol. The section is then washed thoroughly and carefully with xylol and mounted in xylol balsam.

When the colour of a series of sections is insufficiently deep, one can intensify the staining by leaving the sections in 5 per cent. oxalic acid for twenty-four to forty-eight hours after Flemming's solution. During the procedure of staining and differentiation a section often disappears.

Losing a section from a slide may seem rather ridiculous, but, when one realises how deeply Victoria blue stains everything it comes in contact with, it is quite understandable. The worker should proceed very carefully, and after he pours off the stain he should hold the slide up to the light; by doing this it is easy to see whether the section is there or not. In spite of the difficulties the method can be recommended.

Résumé

(1) Fix for 15 days or more in 10 per cent. formalin.
(2) Cut frozen sections at 15-20μ.
(3) Wash several minutes in distilled water.
(4) Place in a saturated solution of mercuric chloride for 2 hours at laboratory temperature.
(5) Without washing transfer to modified Flemming's solution for 24-48 hours at laboratory temperature.
(6) Place cigarette paper on slide and wet with distilled water.
(7) Wash a section rapidly in distilled water.
(8) Transfer to a dish of distilled water and float it on to the paper on the slide.
(9) Pour 1·5 per cent. Victoria blue on the section.
(10) Heat gently over the by-pass flame until steam rises.
(11) Repeat No. 10 for at least 3 times.
(12) Remove the stain and allow the slide to cool.

(13) Pour Lugol's iodine over the section and leave 1 minute.
(14) Throw off the iodine and differentiate as described in detail.
(15) Transfer the section to another slide.
(16) Wash carefully and thoroughly in xylol and mount in xylol balsam.

Gold Sublimate Method for Protoplasmic (Cortical) Neuroglia. (Ramon-y-Cajal.)

This method is the easiest of all neuroglia methods and cannot fail if the worker follows the instructions given. Pieces of tissue 5 mm. thick are placed as soon as possible after death in formol bromide solution for two to eight days. Good results are obtained from tissue left in this fixative for rather longer periods. After this the staining of "protoplasmic" neuroglia does not appear to be so good, but the "fibrous" neuroglia is still stainable by this method for some weeks.

Frozen sections are cut at 20μ and are transferred to formol bromide until ready for staining. They are then washed in distilled water and transferred to Cajal's gold sublimate for four to eight hours in the dark at a temperature of 22° C. If the worker has an incubator working at this temperature it will be to his advantage, as the laboratory temperature in this country is rarely so high; otherwise it may be necessary

for the section to be kept longer in the gold. There should not be more than seven to eight sections to every 30 c.c. of the stain, and they must be spread out flat on the bottom of the dish to obtain an even staining. The sections must take on a deep purplish-brown colour before they are taken from the gold bath. Once this colour is obtained they are washed quickly in plenty of distilled water and transferred to 5 per cent. sodium hyposulphite containing 10 per cent. absolute alcohol for five to ten minutes. The sections are then washed in water and taken through two changes of 40 per cent. alcohol, floated on to clean slides, properly dehydrated, cleared and mounted in xylol balsam.

Experience has shown that it is quite unnecessary to use the 40 per cent. alcohol baths, the alternative being to pass them from hyposulphite into a dish of distilled water; a section is then floated on to a clean slide, blotted, dehydrated, cleared and mounted.

Résumé

(1) Fix pieces 5 mm. thick in formol bromide 2-8 days.
(2) Cut frozen sections at 20μ.
(3) Transfer to formol bromide until ready for staining.
(4) Wash and stain in gold sublimate 4-8 hours in the dark at 22° C.
(5) Wash quickly in distilled water and fix in 5 per cent. hyposulphite 5-10 minutes.

(6) Wash and pass through 2 changes of 40 per cent. alcohol.

(7) Float a section on to a clean slide, blot, dehydrate, clear and mount in xylol balsam.

Microglia—Del Rio-Hortega's Method

(1) Small pieces of tissue must be fixed within a few hours of death in formol-bromide solution. They should remain in the fixative for not less than one and not more than four days.

(2) Heat the block in fresh formol-bromide solution for ten minutes in the paraffin oven at 50° C. Cut frozen sections at 20 to 30μ, receiving them in distilled water to which a little ammonia has been added (eight drops for each 100 c.c.).

(3) After a rapid wash in distilled water the sections are transferred to Hortega's silver carbonate solution (*see* page 135), in which they remain, either at room temperature or in the incubator or paraffin oven until they are a light yellow colour. The time and heat required for this vary with different pieces of tissue.

(4) Without washing reduce in 1 per cent. formalin, keeping the sections moving in this, for one minute. The sections should then be greyish-yellow in colour.

(5) Wash, tone in gold chloride, fix in hyposulphite, wash again, dehydrate, clear and mount in xylol balsam.

As a variant of this method the sections may

be cut without previous warming of the block and transferred directly to fresh formol bromide solution, in which they are heated to 50° C. for ten to fifteen minutes. If the sections turn a dark yellow colour they may be treated, *after washing*, with stronger solutions of formalin.

Cone and Penfield's Modification of Hortega's Microglia Method

This modification makes the staining of microglia much easier, especially in normal tissue, and in tissue which has been fixed in formalin or which has remained in formol bromide solution for more than the optimum time.

(1) Cut sections of formalin fixed tissue by the freezing method at 20μ.

(2) Place sections in weak ammonia in distilled water overnight.

(3) Transfer directly to 5 per cent. hydrobromic acid at 37° C. for one hour.

(4) Wash in three changes of water.

(5) Place in 5 per cent. sodium carbonate for one hour (up to five or six hours).

(6) Pass sections with or without washing into

10 per cent. silver nitrate .	5 c.c.
5 per cent. pure sodium carbonate . . .	20 c.c.
Ammonia to dissolve ppt. .	
Distilled water to . .	75 c.c.

for three to five minutes, when they remain uncoloured, or longer till they turn light brown.

(7) Place in 1 per cent. formalin and agitate.
(8) Wash in distilled water.
(9) Leave in 1 in 500 gold chloride till sections turn a smooth bluish-grey.
(10) Fix in 5 per cent. sodium hyposulphite.
(11) Wash, dehydrate, clear, and mount.

Scharlach R. (Herxheimer)

Staining with Scharlach R. is very simple and can be used for sections of the brain and cord, as well as for most internal organs. Frozen sections are cut at 16-20μ, washed in distilled water, and then transferred to 70 per cent. alcohol for five to ten minutes. From this they are put into a saturated solution of Scharlach R. in equal parts of 70 per cent. alcohol and of acetone for three to five minutes, or sometimes longer. After this they are differentiated in 70 per cent. alcohol until they show a nice pink colour and then transferred into distilled water.

The sections are now counter-stained in alum hæmatoxylin. This should be just enough to show the nuclei ; if Anderson's alum hæmatoxylin is used, one to one and a half minutes is quite long enough.

N.B.—If the hæmatoxylin staining is prolonged until the myelin sheaths are stained by it, it is impossible to differentiate them with acid alcohol.

Sections are now washed in tap water until the nuclei have had time to become blue. They are then mounted in glycerine jelly. When mounting sections in this mountant, air bubbles very frequently appear in the sections. Trying to remove them by pressing down the cover slip invariably destroys the section. The best way is to have a pipette, with a rubber teat, standing in the jelly while it is being dissolved. When the section is to be mounted sqeeze out the first drop or two of jelly from the pipette and use the subsequent drops for mounting; drop one on the coverslip and another on the section; by doing this most of the bubbles are eliminated.

A stain containing equal parts of Grübler's and Gurr's Scharlach R. gives excellent results. It is advised when staining to keep the section in a closed bottle, as acetone quickly evaporates and a precipitate may be thrown down.

Résumé

(1) Cut frozen sections at 16-20μ.
(2) Wash in distilled water.
(3) Transfer to 70 per cent. alcohol 5-10 minutes.
(4) Stain in Scharlach R. for 3-5 minutes.
(5) Differentiate in 70 per cent alcohol.
(6) Wash in distilled water.
(7) Counter-stain in alum hæmatoxylin 1-1½ minutes.
(8) Wash in tap water and mount in glycerine jelly.

CHAPTER IV

PARAFFIN SECTIONS AND SOME SPECIAL METHODS IN WHICH THEY ARE USED

It has already been stated that paraffin embedding is not satisfactory for the nervous system owing to the shrinkage which invariably takes place during this process, yet it is impossible to avoid paraffin altogether; therefore it is best to use certain precautions by which shrinkage may be reduced.

A certain amount of shrinkage is sure to take place when the tissue is in the alcohols; this is unavoidable, but it may safely be stated that the greatest amount takes place during clearing in xylol and passing through the paraffin bath, therefore the chief point is to clear in reagents which affect the tissue less and allow of the infiltration of paraffin in the shortest possible time. The shrinkage of tissue is reduced if it is cleared in cedarwood oil and then washed well in benzol before being put into the paraffin bath. Clearing in cedarwood oil takes much longer than in xylol, but the result is worth the time lost. Paraffin used for nervous tissue should not have a melting-point of more than 50° C. The worker will find

a difficulty in cutting sections in a warm room with such paraffin-wax owing to the sections sticking to the knife, but this difficulty is overcome if, after fastening the paraffin block on the object-holder, it is placed in the ice chest until it is chilled through.

Dehydration

Small pieces of tissue taken at operation may, after twenty-four hours in formol saline or Bouin's fluid, be placed in spirit for a few hours and then left overnight in absolute alcohol. If the pieces of tissue are large and numerous the alcohol should be changed and left for another twenty-four hours.

Clearing

If xylol is used for clearing, the tissue should not stay in it longer than is absolutely necessary. When cedarwood oil is used the tissue is placed in it and left there until the tissue has sunk to the bottom of the bottle. The tissue is then thoroughly washed in benzol and passed through the paraffin bath.

Infiltration

Small pieces of tissue should not take more than one and a half hours for infiltration, but for larger pieces the time must be varied. A good plan is to have two sets of paraffin baths in the oven, one set on the dividing shelf and the other underneath the shelf. When the tissue is ready for

the oven it is put into the paraffin on the shelf for a third of the time allowed for the whole process; it is then transferred to the paraffin underneath for the remainder of the time. If this method is adopted there is no need to keep spare pots of paraffin for embedding, the tissue being embedded in the same paraffin it is in last. If paraffin of two different melting-points be bought, *i.e.* 45° C. and 56° C. any melting-point between the two may be obtained by mixing them in proportion. Gurr's paraffin is satisfactory.

Embedding

When embedding the tissue, the paraffin is poured into the mould and the tissue placed in it with a pair of warm forceps. The whole must be chilled rapidly, otherwise the paraffin will become crumbly and the tissue will have to be embedded afresh. After it has become hard the spare paraffin is trimmed off. The top and bottom edges must not be trimmed too close to the tissue and must be perfectly parallel, this being essential when serial sections are to be cut. The side edges are not so important, but if they are cut at equal distance from the tissue, and the nearer the better, the ribbons will come off straight.

Microtomes

There are several different patterns of paraffin microtome on the market of which the rocking

microtome, made by the Cambridge Scientific Instrument Co., is in most common use, but it cuts with a slight curve and only takes blocks of very limited size. These disadvantages make it unsuitable for serial sections of the brain stem.

The Minot is a flat-cutting machine, and fairly large serial sections can be cut with it perfectly well, but this machine needs considerable attention or the sections will vary in thickness.

A very simple and satisfactory flat-cutting microtome is the No. 3 Automatic Rotary Microtome, made by the Laboratory Equipment Co., Regent Street, London, W.

Knives

Whichever machine is used, the best knife to use is one which has a heavy back and is only slightly hollow ground.

Sharpening the knife.—The paraffin knife is not sharpened in the same way as the celloidin knife ; it is drawn across the hone from heel to toe, turned over on its back and drawn in the opposite direction from heel to toe, always moving the knife with its edge forwards. This is repeated until a satisfactory edge is obtained. The stropping is exactly the same as described for the celloidin knife.

Cutting Sections

Before actually cutting sections, the corners of the paraffin block should be slightly trimmed off ;

this ensures the worker getting a dividing line between each section. If the knife is in good condition and the tissue properly infiltrated, ribbons of sections of 4μ may be cut easily, and as they are cut they are laid out on a sheet of black paper, such as is used for packing X-ray films. This is pinned down on the cutting bench in several layers, then as the top layer gets dirtied with bits of old section it may be torn off, leaving a clean one underneath.

Mounting Sections

Paraffin sections from nervous tissue should always be mounted on albuminised slides. Egg albumen is used, and the method of use has been explained in the neuroglia method on frozen sections. The sections are mounted in the following way.

Each section is separated with a sharp knife and placed separately on the surface of a dish of warm water, the temperature being about 10° C. lower than the melting-point of the paraffin used. For sections of the cord water with an even lower temperature than this is advised. As the sections are placed on the surface of the water the creases usually come out immediately, but sometimes it is necessary to help this with a pointed knife and a small brush. It is unwise to mount sections from nervous tissue on a slide

with cold water and spread them out over a Bunsen-burner.

When the sections have spread out evenly on the surface of the water they are floated singly on to a slide by dipping the albuminised slide in the water underneath a section. The section is held in place with a small brush and the slide is drawn from the water in an almost upright position. As the sections are mounted they are placed on a tray and put into an incubator at 37° C. until they are perfectly dry. If the worker starts staining before the sections are dry they will not stay on the slide.

If it is necessary to stain the sections as soon as possible the following method is advised :—

Blot the section with fine filter paper directly it has been floated on the slide and place it in the incubator for five minutes ; take it out and flood the section with absolute alcohol and leave for about a minute, pour off the alcohol and dry by waving the slide through the air. When the section is dry the paraffin may be removed with xylol, but care must be taken to see that it is all removed or it will inteferre with the staining. Under ordinary circumstances the sections are left in the incubator overnight.

Staining with Hæmatoxylin

When the paraffin has been dissolved with xylol, the section is washed with absolute alcohol, then

with spirit and then well in water. Paraffin sections are stained in a similar way to celloidin sections except that they take longer to stain. Duplicate sections may be counter-stained with eosin and Van Gieson respectively. It is better to overstain with hæmatoxylin and then differentiate with acid alcohol. After this they are put into tap water.

Counter-Staining

(1) *Eosin.*—After the section has been differentiated it is washed in water and immersed in a dish of tap water to which has been added a few drops of 1 per cent. water-soluble eosin, usually $\frac{1}{2}$ c.c. to every 100 c.c. of tap water. The section is left in this until the red blood corpuscles take on a pink colour. It must be remembered that sections will not take the eosin by this method if the hæmatoxylin is too intense, nor will they do so except in slightly alkaline water. When counter-staining has gone far enough the sections are dehydrated, cleared and mounted in ordinary xylol balsam.

(2) *Van Gieson.*—This is used in the same proportions as given for celloidin, but it is run on the sections from a pipette. The stain is allowed to stay on for six to ten seconds, according to the freshness of the mixture. The slide and section are then washed with spirit, then with absolute alcohol. The section is now flooded with absolute alcohol and left for fifteen to twenty

minutes, after which it is properly dehydrated, cleared and mounted in acid balsam. Iron hæmatoxylin is recommended for staining sections which are to be counter-stained with Van Gieson.

Résumé

(1) Cut sections at 4 to 6μ (8-10μ for serial Nissl sections).
(2) Spread sections out singly in dish of warm water.
(3) Mount on albuminised slide.
(4) Place in incubator to dry.
(5) Dissolve paraffin with xylol.
(6) Wash with absolute alcohol.
(7) Wash with spirit.
(8) Wash well with water.
(9) Stain with hæmatoxylin.
(10) Differentiate with acid alcohol and place in tap water.
(11) Counter-stain with eosin or Van Gieson as described.
(12) Dehydrate, clear and mount, eosin counter-stained sections with ordinary xylol balsam; Van Gieson counter-stained with acid balsam.

Neuro-Fibrils.—Ramon-y-Cajal's Method

Fix small pieces of tissue for twenty-four to forty-eight hours in :—

96 per cent. alcohol . .	100 c.c.
Ammonia ·880 . . .	0·25 c.c.

Wash rapidly in distilled water, and transfer to 3 per cent. silver nitrate solution for five to six days in the incubator at 37° C.

Wash rapidly in distilled water.

Reduce in the following mixture for twenty-four hours in the dark at room temperature (two stock solutions are prepared and kept separately) :—

Sol. A.	Sod. sulphite. .	5 grammes
	Formol . .	50 c.c.
	Distilled water .	200 c.c.
Sol. B.	Pyrogallic acid .	20 grammes
	Distilled water .	800 c.c.

For use take 25 c.c. of A and 80 c.c. of B.

Wash for a few minutes in distilled water and embed in paraffin or celloidin. Cut thin sections.

After the sections have been passed into distilled water they are placed in a combined toning and fixing bath, where they remain for a few minutes until they become of a grey-violet colour.

Toning and fixing bath :—

Ammonium sulpho-cyanide .	3 grammes
Sodium thiosulphate . .	3 grammes
Distilled water to . .	100 c.c.

Add just before use a few drops of 1 per cent. gold chloride.

Wash, dehydrate, clear and mount in Canada balsam.

Bielschowsky's Method of Staining Nerve Fibres in Blocks of Tissue

This method is valuable for any tissue which is too soft for frozen sections to be cut from it, for example, softened areas of cord or brain and the retina. In using it the piece of tissue to be stained should be as thin as possible, as impregnation with silver never penetrates very deeply. In the case of the retina, a small piece of retina is stained in the same way as a block of tissue and is then cut between layers of liver tissue in the manner to be described. Otherwise the method for the retina is the same as for other more solid nervous tissues.

The tissue must be fixed in 10 per cent. formolin or formol saline for eight to ten days at least. Thin pieces are then placed without washing in pure pyridin for two days. The tissue is then washed in running water for twenty-four hours after which it is washed for a few minutes in distilled water. After washing, it is placed in 3 per cent. silver nitrate for four to five days in the dark or in the incubator at 37° C. for two to three days. When the tissue has been in the 3 per cent. silver the requisite time it is washed quickly in distilled water and transferred to Da Fano's ammoniacal silver for four to five hours, then washed again in distilled water and put into 20 per cent. formalin for twelve hours, after which it is transferred to water for a good wash. (To embed retina for

sectioning a piece of well-fixed liver tissue about 4 mm. thick is taken and almost sliced in two. The retina is sandwiched between the two layers, and the whole is tied round with very fine thread. The liver and retina are dehydrated, cleared, and passed through the paraffin in the usual way.) Sections are cut and mounted on slides, as already described.

When the paraffin has been dissolved from the sections they are washed in distilled water and toned in weak gold chloride in the manner explained for frozen sections, fixed in hyposulphite, washed, dehydrated, cleared and mounted in xylol balsam.

Résumé

(1) 10 per cent. formalin 8-10 days.
(2) Pure pyridin 2 days.
(3) Wash in running water 24 hours, then distilled water.
(4) Transfer to 3 per cent. silver nitrate 4-5 days in the dark.
(5) Wash in distilled water and transfer to ammoniacal silver 4-5 hours.
(6) Wash in distilled water and transfer to 20 per cent. formalin for 12 hours.
(7) Wash well in water.
(8) (Sandwich retina between pieces of liver.)
(9) Spirit several hours.
(10) Overnight in absolute alcohol.
(11) Clear in xylol.
(12) Pass through paraffin baths and embed.

(13) Cut sections at 4-6μ
(14) Mount on albuminised slides and dry.
(15) Remove paraffin with xylol.
(16) Wash with absolute alcohol, then with spirit.
(17) Wash in distilled water and tone in weak gold chloride.
(18) Quick wash in distilled water.
(19) Fix in 5 per cent. hyposulphite.
(20) Wash, dehydrate, clear and mount in ordinary balsam.

Neuroglia.—Anderson's Victoria Blue Method for Paraffin Sections

Pieces of formol saline fixed tissue are placed in Bouin's fluid overnight at laboratory temperature. The tissue is then washed in tap water and embedded in paraffin in the usual way. Sections are cut at 6μ, mounted and dried, the paraffin removed, and the sections washed in absolute alcohol, spirit and then warm water until the picric acid is completely removed. After washing the section is flooded with Anderson's neuroglia mordant and left for fifteen to twenty minutes. The mixture is then poured off and the section is washed quickly with distilled water and flooded with 0·25 per cent. potassium permanganate for five to ten minutes. The permanganate is poured off and replaced with Pal's solution ; this is left on until the section is perfectly white. The section is

washed quickly with distilled water, blotted with fine filter paper, and stained with hot 1·5 per cent. Victoria blue for three to five minutes. Further procedure is the same as given in Anderson's Victoria blue method for frozen sections. This method is invaluable for tissue which cannot be cut on the freezing microtome.

Résumé

(1) Place tissue in Bouin's fluid overnight.
(2) Wash in water.
(3) Pass through the paraffin bath and embed in the usual way.
(4) Cut sections at 6-10μ.
(5) Mount on albuminised slides and dry.
(6) Remove paraffin with xylol.
(7) Wash with alcohol, spirit, and then water.
(8) Flood with neuroglia mordant 15-20 minutes.
(9) Quick wash with distilled water.
(10) Permanganate 5-10 minutes.
(11) Pal's solution until section is white.
(12) Quick wash with distilled water and blot.
(13) Stain with hot 1·5 per cent. Victoria blue 3-5 minutes.

Further procedure already described in frozen section method.

Fuchsin Light Green Method for Neuroglia. (Alzheimer)

Small pieces of tissue 5 mm. in thickness are put into 10 per cent. formalin, as soon after

death as possible, for twenty-four hours. The tissue is then transferred to Flemming's solution for eight days, or to a solution containing 18 c.c. of 1 per cent. chromic acid and 2 c.c. of glacial acetic acid. After the tissue has been in the solution the required time, it is washed in running water for twenty-four hours, then dehydrated, cleared in xylol, and embedded in paraffin with a melting-point of 58° C. The sections are cut at 2-3μ, and will cut much easier if the object-holder with the paraffin block fastened on it is put into the ice chest until the paraffin block is chilled through. The sections when cut are mounted on slides, as previously recommended.

The paraffin is removed from the section, and the section is washed in 96 per cent. alcohol. It is then placed in a saturated solution of acid fuchsin and stained for one hour in the paraffin oven at 58° C. The section is then washed in two changes of water until no more stain comes off. It is then placed in the following solution :—

Saturated alcoholic solution of picric acid, 30 c.c.
Distilled water . . . . 60 c.c.

for ten to twenty seconds, the stain to be agitated the whole time. After this stain the section is washed carefully in two changes of distilled water and stained for twenty to fifty minutes in a half-saturated solution of light green. It is

then washed quickly in distilled water, 95 per cent. alcohol, absolute alcohol, cleared in xylol and mounted in ordinary balsam.

Résumé

(1) Fix small piece 5 mm. thick in 10 per cent. formalin for 24 hours.
(2) Flemming's solution 8 days.
(3) Wash in running water for 24 hours.
(4) Dehydrate, clear and embed as usual.
(5) Cut sections at 2-3μ, and mount as advised.
(6) Remove paraffin and wash in 95 per cent. alcohol.
(7) Stain for 1 hour in saturated acid fuchsin in the paraffin oven.
(8) Wash in two changes of distilled water.
(9) Alcoholic picric acid solution 10-20 seconds.
(10) Wash carefully in two changes of distilled water.
(11) 20-50 minutes in $\frac{1}{2}$ saturated solution light green.
(12) Wash quickly in water, 95 per cent. alcohol, absolute alcohol.
(13) Clear in xylol and mount in ordinary balsam.

Paraffin Sections that Will not Stick on the Slide

Paraffin sections of nervous tissue must be mounted on albuminised slides to avoid trouble when staining them. Sometimes, but fortunately not often, the sections tend to come off even when albuminised slides are used. This possi-

bility is rather inconvenient if the worker is doing serial sections, and occurs mostly with alcohol fixed tissue. It usually happens after the paraffin has been removed and the sections are washing in water. If they came off whole they could be preserved, but usually they come off in fragments. Should the worker be doing serial sections, and they should tend to come off the slide, he is advised to take no further risk, but to adopt the following plan. After removing the paraffin, wash the section well in alcohol, blot and immediately pour a very thin solution of celloidin over the whole slide. Most of the celloidin is drained off and the slide left for the celloidin to set. The worker must see that the celloidin does not get too dry or the section will suffer. When the celloidin has set sufficiently, the whole slide is washed in water, and the staining is carried on as though nothing had happened. The celloidin in no way hinders the staining, but clearing must be done in the manner explained for celloidin sections. This method obviates all risk of further sections coming off the slide.

CHAPTER V

SPECIAL METHODS FOR STAINING FAT, IRON, AND CALCIUM

The following methods were thoroughly worked out and used extensively at the National Hospital, Queen Square, by Dr. E. Weston Hurst, by whose kindness in transcribing them they can now be published in detail.

Fat-Staining Methods

For the detection of the different lipoid substances various methods must be used, since no one method at present in use reveals all lipoids present. Advantage is taken of this fact in the identification of these fatty substances, but since the number of fat-staining methods is comparatively small and their selectivity only partial, other means must be adopted for the complete identification of the lipoids, especially where, as is often the case, several exist together in the same cell or globule. Such means consist in the determination of the solubilities of the lipoids and of their reaction to polarised light.

For routine examination, Scharlach R. and

Sudan III applied to frozen sections are undoubtedly the most useful dyes. The method used with the former stain, which is to be recommended, has already been given, and by its agency neutral fats, cholesterin-esters and cholersterin-fatty acid mixtures, which play an important part in nervous degeneration, can be revealed.

Marchi's Method for Frozen Sections

Osmic acid, though reacting in some degree with all fatty substances, is not good as a routine stain for nervous tissue, for not only do the sections become extremely brittle, but staining of the myelin sheaths renders microscopical examination difficult. It may be used as a 1 per cent. solution in which frozen sections are immersed for twenty-four hours after a thorough preliminary washing in water. It is commonly stated that fat, after treatment with osmic acid, resists solution in chloroform balsam, but, in practice, perhaps as the result of imperfect penetration of the globules, some of the lipoids disappear when mounted in this medium.

If immediate examination is contemplated, glycerine jelly is a suitable mountant, but in the course of a few days the osmic acid causes it to become a dark greenish yellow and the preparation is no longer of any value. After preliminary treatment with potassium bichromate only the

neutral fats, fatty acids and lipochrome pigments become blackened by osmic acid. This fact forms the basis of Marchi's method, the application of which to blocks of tissue has already been described. It may be used also for frozen sections as follows :—

(1) Wash thoroughly in distilled water.

(2) Transfer to 2 per cent. potassium bichromate for twenty-four hours.

(3) Wash thirty minutes in several changes of distilled water.

(4) Immerse in 1 per cent. osmic acid for twenty-four hours.

The above remarks as to mounting apply equally to this method.

Nile Blue

Lorrain Smith (*Jour. of Path. and Bact.*, 1908 xii, 1) showed that by the use of this dye it is possible to distinguish between fatty acids and neutral fats, the former assuming a blue colour, while the latter stains pink. A mixture of these bodies becomes purple, the exact tint depending on their selective concentration. Other lipoid bodies, however, *e.g.* the myelin sheaths and lipochrome pigments, assume a blue colour, and the value of the method lies, therefore, in its ability to demonstrate the presence of neutral fats.

(1) Formalin fixed material is cut by the

freezing microtome and washed thoroughly in distilled water.

(2) Immerse sections for ten minutes in a saturated solution of nile blue sulphate.

(3) Wash a few minutes in distilled water.

(4) Mount in glycerine jelly.

The preparations do not keep more than a few days.

Progressive Mordanting by the Weigert Pal Method and its Modification

Lorrain Smith and Mair (*Jour. of Path. and Bact.*, 1909, xiii, 14) pointed out that, by this method, the various fatty substances stain after different periods of mordanting. The Kultschitsky Pal method for frozen sections already given has the advantage that a comparatively short time in the mordant is sufficient.

Normal myelin stains well after twenty-four to forty-eight hours mordanting at 37° C., while if mordanting be continued for ninety-six hours the staining is not nearly so intense. Lipochrome pigments, on the other hand, stain feebly after one and two days, and cholesterin not at all.

Klotz Method for Fatty Acid (*Jour. of Exp. Med.*, 1906, viii, 322)

This is an application of the above-mentioned principle to the demonstration of a particular lipoid.

STAINING FAT, IRON, CALCIUM

(1) Fix tissue for one to twenty-four hours in a solution of :—

chromogen . . .	2·5 grm.
4 per cent. formalin . .	100 c.c.

Boil, and while cooling add acetic acid 5 c.c., copper acetate (powdered) 5 gm.

(2) Wash thoroughly in distilled water.

(3) Cut sections on freezing microtome, or alternatively frozen sections may be cut from formalin fixed material and immersed in the above solution for one hour at 37° C.

(4) Stain in a saturated solution of hæmatoxylin in 60 per cent. alcohol one hour at 37° C.

(5) Transfer to water.

(6) Decolourise until tissue becomes a light brown with :—

Potassium ferricyanide .	2·5 grm.
Borax	2 grm.
Distilled water . . .	100 c.c.

(7) Mount in glycerine jelly.

Solubility Tests

These are carried out by immersing the sections over-night in the desired solvents. The solvents usually used are acetone, alcohol and chloroform. All of these remove neutral fats, cholesterin and fatty acids. Alcohol, in addition,

removes the phosphatides, and chloroform all fatty substances except lipochrome pigment.

Since sections cannot be taken directly into chloroform from water, or into absolute alcohol, without becoming wrinkled and distorted, it is better to proceed as follows :—

(1) Place sections in 70 per cent. alcohol.

(2) Transfer one-third into acetone, the remainder into 90 per cent. alcohol.

(3) Transfer half of those in 90 per cent. alcohol into chloroform.

The fact that the sections now in chloroform have been taken through alcohol does not matter, as any lipoid which will resist the action of chloroform will equally be insoluble in alcohol.

Ciaccio (*Pathologica*, 1921, xiii, 183) has shown that fatty acids of the saturated series resist solution in the usual solvents after mordanting with zinc acetate. Sections may be immersed in a saturated solution of zinc acetate for twelve to twenty-four hours, and rapidly washed in water before being transferred into alcohol. If after this solvent any fatty acid remains in the section it may be confused with lipochrome pigment and the cerebrosides, both of which normally are insoluble in alcohol.

Ciaccio recommends that the cerebrosides be removed with a mixture of methyl alcohol and chloroform in equal parts, thus removing one difficulty. In distinguishing between lipochrome pigments and the mordanted fatty acids we

take advantage of the fact that the latter are removed by an acidified mixture of equal parts of absolute alcohol and ether.

Double Refraction

The final method for the investigation of the fats is the determination of their reaction to polarised light. Neutral fats, fatty acids and lipochrome pigment are not doubly refractile. The other lipoids are doubly refractile to varying extents. The exact technique cannot be described here, but the apparatus consists in a fixed Nicol prism placed beneath the stage of a microscope and a rotating prism placed in the eyepiece.

Method for Staining Iron and Calcium.—Staining for Iron

The well-known chemical reaction between ferric salts and potassium ferrocyanide in the presence of hydrochloric acid (Prussian blue reaction) may be applied to pieces of tissue as a macroscopic test, or to sections as a microscopic test, for the presence of iron.

The reagents used consist of a mixture of one part of 2 per cent. potassium ferrocyanide (aqueous solution) and one part of 2 per cent. strong hydrochloric acid, and it is applied for fifteen to thirty minutes. For sections this method has one grave disadvantage, viz. that spontaneous precipitation of blue pigment may

occur, and though this is said to be avoidable by the use of excess of acid as in the above formula, it nevertheless does undoubtedly still occur.

A similar reaction (Turnbull's blue reaction) indicates the presence of ferrous salts ; potassium ferricyanide is substituted for the ferrocyanide, and does not have the same tendency to the precipitation of blue pigment. Perhaps the best method, however, is that of employing ammonium sulphide, which reacts with both ferrous and ferric salts.

Frozen or celloidin sections are equally suitable, but the latter must have the celloidin removed by placing them in equal parts of alcohol and ether. This method for removing celloidin from sections is fully explained in the method for staining Nissl bodies.

(1) If celloidin sections are used, remove the celloidin as advised and wash in distilled water.

(2) Immerse overnight in a fresh concentrated solution of ammonium sulphide ; some workers recommend that this be not less than three days and not more than three weeks old.

(3) Wash thoroughly in distilled water, the iron is now converted to the greenish black sulphide which is somewhat difficult to recognise microscopically, and it is better to proceed to the next stage.

(4) Wash in several changes of distilled water.

(5) Tone for half to one hour in a mixture of potassium ferricyanide and hydrochloric acid, prepared as indicated above.

(6) Wash thoroughly in four or five changes of distilled water; neglect of this precaution will cause an unsightly precipitation of the counter-stain.

(7) Counter-stain for three minutes in 1 per cent. watery neutral red.

(8) Dehydrate, clear in xylol and mount in Canada balsam.

N.B.—The staining of the iron tends to fade in several months.

Macallum's Method (*Jour. of Physiol.*, 1897)

This method is extremely sensitive. Sections are stained for ten minutes in a $\frac{1}{2}$ per cent. solution of pure hæmatoxylin in perfectly pure distilled water (the solution being of a pale brownish yellow colour). The sections are washed for fifteen to thirty minutes in a mixture of equal parts of absolute alcohol and ether to remove the stain from the tissue, washed in water and counter-stained as above. It should be remembered that fixation in formalin tends to remove the iron from the tissue, and though short fixation in this fluid may do little damage, the iron will all be removed if left in it for a long time. Alcohol does not have this disadvantage, therefore on the whole is preferable as a fixative for tissue which is going to be stained for iron.

Iron in the tissue may exist in three forms :—

A.—Inorganic iron, which reacts immediately with the methods already mentioned. Mac-

allum's method also stains the albuminate of iron which behaves in this respect like the inorganic compounds.

B.—Organic iron, which may readily be separated from its combination with the tissue elements by the action of acid alcohol for, say, twenty-four hours. Long immersion in acid completely removes the iron ; it is said that nitric acid has less tendency to effect this.

C.—Iron in stable organic combination, e.g. as in hæmoglobin, which cannot be detected by the above methods.

Methods for Staining Calcium

The staining of calcium is a matter of some difficulty as, unfortunately, the methods commonly employed are not specific. Calcified areas stain an intense blue-black colour with alum hæmatoxylin, and black with hæmatoxylin in the Kultschitsky Pal method and its modification. Iron is, however, practically always associated with calcium in these deposits, and it is believed by some that iron is responsible for the reaction with hæmatoxylin. In Rochl's method the sections are placed in a half-saturated solution of oxalic acid to remove the iron, and then stained by a 1 per cent. aqueous hæmatoxylin solution. Even after the complete removal of the calcium by the action of hydrochloric acid, the degenerated material by which it was absorbed gives a deep blue colour with hæma-

toxylin, so that there is added difficulty in the sure detection of calcium.

Jahnel's Method for Staining Spirochætes

As Levaditi's method tends to stain nerve fibres, Jahnel's method is generally used for staining spirochætes in nervous tissue.

1. Put pieces of formalin or alcohol fixed tissue, 2 to 4 mm. thick, into distilled water for one to three days.
2. Transfer to pure pyridin for one day.
3. Wash in many changes of distilled water one to three days.
4. Put into 10 per cent. pure formalin for one to two days.
5. Wash rapidly in water and transfer to 1 per cent. uranium nitrate solution in distilled water for one half to one hour at 37° to 50° C.
6. Wash in distilled water one day.
7. Put into 90 per cent. alcohol three to eight days.
8. Wash in distilled water till tissue sinks.
9. Transfer to freshly made 1·5 per cent. silver nitrate solution for five to eight days at 37° C.
10. Wash quickly in distilled water and "develop" for one to two days in the dark in—

4 per cent. Pyrogallic acid .	95 c.c.
Pure formalin . . .	5 c.c.

11. Wash in distilled water, dehydrate, clear, and embed in paraffin. Cut sections at 10μ.

CHAPTER VI

MISCELLANEA

A.—Cleaning Dirty Celloidin

Celloidin is often contaminated during the process of infiltration, especially of tissue which has been mordanted. This celloidin can be reconditioned for further use by the following process. The solution of dirty celloidin, collected in a stoppered bottle, is filtered through gauze into a large dish. On to this is poured 0·25 per cent. potassium permanganate, mixing all the time. This throws the celloidin out of solution.

When the fluid part loses its slipperiness it is poured off and the residue is transferred into Pal's solution or 5 per cent. oxalic acid, and left in this until it has all turned white. It is then thoroughly washed in several changes of distilled water for twenty-four hours. By this time it will have become hard and shreddy; the water is now poured off and the residue is left to dry at laboratory temperature. When dry it may be dissolved into 2 per cent. celloidin for further use.

Celloidin made by this process will have a

greenish tint, but experience has proved this does not interfere with its usefulness.

N.B.—All bits of clean trimming are thoroughly dried and used for making 8 per cent. celloidin.

B.—Cutting Celloidin Sections from Brittle Material

After pieces of tissue have been through chrome mordants, especially if they are large pieces, they are apt to become very brittle, and it is impossible to cut even very thick sections. To obviate this the best way is to make a thin film of celloidin over the tissue previous to cutting a section. This is done in the following way.

Once it has been realised that the tissue is brittle, the block should be squared up and cut flat preparatory to cutting sections. The spirit is blotted off the tissue and thin celloidin is spread evenly over the surface of the block with a small, soft brush. The celloidin is allowed to dry for a few minutes, and, when it becomes fairly firm, spirit is poured over it until the whole is flooded: this will help to harden the celloidin. After a few minutes the worker can proceed to cut a section as he would do in the ordinary way. With a little practice and patience, sections may be cut in this way as easily as though the tissue were not brittle. When this method is used there is no need for further plating, because the film of celloidin serves the same purpose as when

the sections are plated. The procedure is slow, nevertheless it overcomes a difficulty which is often otherwise insuperable.

C.—Re-embedding

Mordanted tissue has a way of breaking and cracking occasionally after it has been stuck on the wooden blocks. If this should happen it is seldom wise to use the method of coating the tissue with celloidin because, as the knife is drawn through, it is liable to pull up pieces of tissue from below. The following plan is advisable. The tissue should be first taken carefully off the block. This may show that it is in several pieces, therefore the worker must be careful not to lose account of how they fit.

The pieces are re-embedded in a dish of celloidin and pushed as close to each other as possible ; further procedure is the same as the original embedding. When the tissue is again mounted on the block the worker will find he can cut the sections as though nothing had happened. It is obvious that the cracks will show, but this is better than the possible loss of the material.

D.—Mounting Sections for Lantern Slides

Sections which have been stained for myelin sheaths may be mounted on slides and shown by a projection lantern similar to ordinary photographic slides. The only objection to this is that

it takes a long time for balsam to set hard enough for it to stand the heat.

The following method for mounting sections on lantern slides makes it possible for them to be shown a week after they are mounted without any fear of the cover-slip sliding off.

It has already been explained how to plate sections in order and to cut them off the plate in strips of a desired number. Instead of mounting the strips on ordinary slides they are mounted on glass squares $3\frac{1}{4}$ by $3\frac{1}{4}$ inches, using thick Canada balsam. The cover-glass should be No. 2, and not less than 3 by $1\frac{1}{2}$ inches. When the cover-glass has been put on it should be pressed down carefully but firmly, so that all the air bubbles are pressed out. The balsam which is squeezed out is partly cleaned off the slide and the remainder is smeared equally round the edges of the cover-glass with a soft brush. The slide is then placed on a warm slab for twenty-four hours. After this the edges of the cover-glass are painted over with a thin, even coating of best gilder's gold size. In three or four hours another coating of gold size is given and the slide left to dry over-night.

The slide can now be enamelled with black or white, according to taste ; white is the stronger, but black is preferable, as it gives a better appearance when finished. It is best to use a rapid drying enamel, because when the enamel is too long in drying it has a tendency to run

under the cover-glass. "Club black enamel" has proved to be best, but it needs two or three coats before it gives a really black enamel on glass. This enamel is painted on in one direction only, with a good soft brush. It should not be laid on too thick the first time; neither should the brush be pressed too hard. The enamelling should overlap the cover-glass to within a quarter of an inch of the section. When "club black" is used, the second coat may be put on fifteen minutes later. During the second coating the edges of the enamel near the section should be lined in and made straight. This coat may be laid on thicker than the first and usually suffices; if not, another coat may be given as soon as the second one is dry. A word of warning must be given; if there is the smallest air hole in the coating of gold size the enamel will run under the cover-glass and spoil the slide.

When the enamelling has been finished the slide is left on the flat for twenty-four hours. Once the enamel has set hard the slide can be shown on the screen with confidence, and when not in use can be stored away in a lantern slide box. If several slides are made by this method they should never be packed one on top of the other. This method allows actual sections to be shown on the screen, and this is simpler and gives a more exact image than making photographic lantern slides.

APPENDIX

(FORMULÆ)

Acid Alcohol

70 per cent. alcohol . . .	99 c.c.
Hydrochloric acid . . .	1 c.c.

Acid Balsam

Saturate ordinary xylol balsam with salicylic acid crystals by mixing the crystals with the balsam. Leave in the incubator at 37° C. overnight.

Alum (Ammonia or Potash)

Add crystals of ammonia or potash alum gradually to boiling distilled water until no more are dissolved. Cool and allow the crystals which form to deposit at the bottom of the bottle.

Bouin's Solution

Glacial acetic acid	5 c.c.
Formalin (40 per cent. formaldehyde) .	25 c.c.
Picric acid. Saturated watery solution .	100 c.c.

Busch's Fluid

1·5 per cent. sodium or potassium iodate	2 parts
1 per cent. osmic acid	1 part

Calcium Hypochlorite ("chloride of lime")

Calcium hypochlorite .	2 grms.
Distilled water . .	100 c.c.

Shake up the hypochlorite in the distilled water and allow it to stand for 4 hours with occasional shaking. Filter and keep in well stoppered bottle.

Carbol-xylol

Xylol	300 c.c.
Pure white phenol crystals.	100 grms.

Carmine (Alum carmine)

Carmine	1 grm.
2 per cent. calcium hypochlorite . . .	5 c.c.
Saturated solution ammonia alum . . .	90 c.c.
Glacial acetic acid . .	5 c.c.

Preparation.—Put 1 grm. of carmine and 10 c.c. absolute alcohol into a 200 c.c. flask. Mix thoroughly. Add 5 c.c. of hypochlorite solution and mix again. Then add 90 c.c. of ammonia alum solution and shake well. Bring the mixture to the boil shaking several times while it is heating.

Boil for 1 minute and filter. After filtering and cooling add the acetic acid and keep in a well stoppered bottle. The solution keeps indefinitely if not used. It should always be filtered after use.

Chromo-hypochlorite

5 per cent. potassium bichromate	15 c.c.
2 per cent. calcium hypochlorite	5 c.c.

Egg Albumen

Beat up the white of one egg and shake up in 200 c.c. of distilled water. Filter through gauze and add 1 grm. of menthol crystals.

Flemming's Solution

1 per cent. chromic acid	15 c.c.
2 per cent. osmic acid	4 c.c.
Glacial acetic acid	1 c.c.

Formol Bromide

Ammonium bromide	2 grms.
Formalin (pure)	14 c.c.
Distilled water to	100 c.c.

Formol Saline

Sodium chloride	1 grm.
Formalin	10 c.c.
Water to	100 c.c.

Gliabeize (Weigert's)

Fluorchrome (Chromium fluoride)	2·5 grms.
Copper acetate	5 grms.
Distilled water	100 c.c.

Boil, and while boiling, add 3 c.c. glacial acetic acid. Filter and keep in stoppered bottle.

Glycerine Jelly (Kaiser's)

Finest French gelatine	8 grms.
Carbolic acid crystals	5 grms.
Glycerine	50 c.c.
Distilled water	42 c.c.

Mix the water glycerine and gelatine together and put into the water bath at 55° C. until the gelatine is dissolved. Then add the carbolic acid crystals and filter. Allow to cool and set. Melt in a bath of boiling water before use.

Gold Sublimate

Dissolve 2 grms. of mercuric chloride (corrosive sublimate) in 40 c.c. of distilled water by heating gently. Then add 10 c.c. of 1 per cent. gold chloride. Cajal advises the use of Merck's brown gold chloride but we have found this to be unnecessary.

The solution should be made up immediately before use.

Gothard's Solution

Cresote	50 c.c.
Cajuput oil	40 c.c.

Xylol	50 c.c.
Absolute alcohol	160 c.c.

Gum Solution (Obregia)

Syrupus simplex	30 c.c.
Dextrine syrup	20 c.c.
95 per cent. alcohol	20 c.c.

Anderson's modification.—Dissolve 45 grms. of white dextrine in 150 c.c. of syrupus simplex by boiling until a clear solution is obtained. Then add 150 c.c. of 80 per cent. alcohol gradually with constant shaking. Shake well before use.

Hæmatoxylin

(i) Anderson's Acid Hæmatoxylin

Hæmatoxylin crystals	0·25 to 0·5 grm.
Absolute alcohol	5 c.c.
2 per cent. calcium hypochlorite	20 c.c.
Saturated ammonia or potash alum	70 c.c.
Glacial acetic acid	5 c.c.

Shake up the hæmatoxylin in the alcohol, add the calcium hypochlorite, and mix well. Add this brown solution to the alum solution gradually with constant shaking. Then add the acetic acid. The stain is then ready for use. Grübler's hæmatoxylin may be used in 0·25 per cent. strength. Other makes of hæmatoxylin in 0·5 per cent. strength.

(ii) Anderson's Iron Alum Hæmatoxylin

Solution (1) :—

Hæmatoxylin . .	0·5 grm.
50 per cent. alcohol . .	100 c.c.

Shake up well and add 3 c.c. of 2 per cent. calcium hypochlorite.

Solution (2) :—

Iron alum	2 to 4 grms.
Distilled water . . .	100 c.c.
Concentrated sulphuric acid .	2 to 3 c.c.

For use, mix 2 parts of solution (1) and 1 part of (2). The mixture does not keep for more than a few days. It stains very rapidly and it is usually necessary to differentiate with acid alcohol.

(iii) Delafield's Hæmatoxylin

Hæmatoxylin crystals . .	4 grms.
Absolute alcohol . . .	25 c.c.
90 per cent. alcohol . .	100 c.c.
Glycerine	100 c.c.
Saturated ammonia alum solution . . .	400 c.c.

Dissolve 4 grms. of hæmatoxylin in 25 c.c. of absolute alcohol. Add this to 400 c.c. saturated ammonia alum solution. Mix well and expose to light and air for 3-4 days. Filter, and then add 100 c.c. glycerine and 100 c.c. of 90 per cent. alcohol, and filter again.

The mixture may be diluted with distilled water or with ammonia alum solution.

(iv) Ehrlich's Acid Hæmatoxylin

Hæmatoxylin	2 grms.
Absolute alcohol	100 c.c.
Glycerine	100 c.c.
Water	100 c.c.
Potash alum	in excess
Glacial acetic acid	10 c.c.

Allow to stand for a week exposed to air and light and filter. The best staining results are obtained after six months.

(v) Kultschitsky's Hæmatoxylin

10 per cent. hæmatoxylin in absolute alcohol allowed to ripen for some weeks	10 c.c.
Distilled water	90 c.c.
Glacial acetic acid	2 c.c.

(vi) Modified Kultschitsky Hæmatoxylin

Hæmatoxylin crystals	0·5 grm.
Absolute alcohol	10 c.c.
2 per cent. calcium hypochlorite	3 c.c.
Mix and then add	
Distilled water	84 c.c.
Glacial acetic acid	3 c.c.

(vii) Phosphotungstic Acid Hæmatoxylin (Mallory)

Hæmatein ammonium	0·1 grm.
Distilled water	100 c.c.
Phosphotungstic acid crystals	2 grms.

Dissolve the hæmatein in a little water by the aid of heat. After it is cool add water up to 100 c.c. Then add the acid.

The stain is ripened by adding 5 c.c. of $\frac{1}{4}$ per cent. potassium permanganate solution.

Hæmatoxylin crystals may be used instead of hæmatein, but it requires 10 c.c. of $\frac{1}{4}$ per cent. permanganate to ripen it.

(viii) Weigert's Iron Hæmatoxylin

Solution (1) :—

Hæmatoxylin	1 grm.
90 per cent. alcohol	100 c.c.

Solution (2) :—

Perchloride of iron	0·4 grm.
Distilled water	100 c.c.
Hydrochloric acid	1 c.c.

Mix equal parts of (1) and (2) just before use. The hæmatoxylin should not be very ripe.

(ix) Weigert's Lithium Hæmatoxylin

Hæmatoxylin	0·75 grm.
Distilled water	100 c.c.

Dissolve the hæmatoxylin by heat. After cooling add 10 c.c. absolute alcohol. When using the stain add 2 c.c. of a saturated solution of lithium carbonate.

Iodine (concentrated)

Iodine crystals	1 grm.
Potassium iodide	2 grms.
Distilled water	100 c.c.

Iodine (Gram's)

Iodine crystals . . .	1 grm.
Potassium iodate . .	2 grms.
Distilled water . . .	300 c.c.

Iodine (Lugol's)

Iodine crystals . . .	1 grm.
Potassium iodate . .	2 grms.
Distilled water . . .	200 c.c.

Marchi's Fluid

Muller's fluid . . .	2 parts
1 per cent. osmic acid . .	1 part

Muller's Fluid

Potassium bichromate . .	2·5 grms.
Sodium sulphate . . .	1 grm.
Distilled water . . .	100 c.c.

dissolve by heating.

Neuroglia Mordant (Anderson's)

(i)	Distilled water . . .	100 c.c.
	Sodium sulphite . . .	5 grms.
	Oxalic acid	2·5 grms.
	Potassium iodide . .	5 grms.
	Iodine crystals . . .	2·5 grms.

Dissolve in the above order and then add 5 c.c. of glacial acetic acid and keep in a well-stoppered

bottle. If it turns brown add 1 or 2 crystals of sodium sulphite and shake.

(ii) Ferric chloride solution, 5 per cent.

For use, mix equal parts of solutions (1) and (2).

Pal's Solution

Oxalic acid	1 grm.
Sodium sulphite . . .	1 grm.
Distilled water . . .	200 c.c.

Scharlach R. (Herxheimer)

70 per cent. alcohol .	50 c.c.
Acetone . . .	50 c.c. Mix

Add Scharlach R. to saturation. Allow to settle and use decanted clear fluid.

Silver Baths

(i) Bielschowsky's Ammoniacal Silver Oxide

20 per cent. silver nitrate .	5 c.c.
40 per cent. sodium hydrate .	6 drops

Add ammonia drop by drop shaking constantly till the precipitate is just dissolved. Make up to 25 c.c. with distilled water.

(ii) Da Fano's Ammoniacal Silver Oxide

20 per cent. silver nitrate .	5 c.c.
40 per cent. sodium hydrate .	2 drops

Add ammonia drop by drop till precipitate is just dissolved. Make up to 40 c.c. with distilled water.

(iii) Hortega's Silver Carbonate Solution

10 per cent. silver nitrate	5 c.c.
5 per cent. sodium carbonate	30 c.c.

Dissolve the precipitate in strong ammonia and dilute to 150 c.c. with distilled water.

Van Gieson's Stain

Solution A.

Distilled water	100 c.c.
Dry picric acid crystals	0·6 grms.

Solution B.

Acid fuchsin	1 grm.
Solution A	100 c.c.

For use, add to 1 c.c. of solution B 10 to 20 of solution A.

Weigert's Differentiator

Potassium ferricyanide	2·5 grms.
Borax	2 grms.
Distilled water	100 c.c.

Weigert's Primary Mordant

Potassium bichromate	5 grms.
Fluorchrome (Kahlbaum)	2·5 grms.
Water	100 c.c.

Add the bichromate to the water and bring to the boil. Then add the fluorchrome. Stir well. Cool and filter.

This mordant may be modified by the addition of 10 c.c. of 2 per cent. calcium hypochlorite to 90 c.c. of mordant.

Zenker's Solution

Mercuric chloride . .	5 grms.
Sodium sulphate . . .	1 grm.
Potassium bichromate .	2·5 grms.
Water	100 c.c.

Dissolve with heat and keep as a stock solution. Add 5 c.c. glacial acetic acid just before use.

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